PATENT
CUSTOMER No. 32425

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Johan FROSTEGARD	Group Art Unit: 1641
Serial No.: 10/814,125	Examiner: LISA V. COOK
Filed: April 1, 2004	Atty. Dkt. No.: EPCL:010US
For: METHOD OF DIAGNOSING CARDIOVASCULAR DISEASE	Confirmation No.: 8029

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September 12, 2007
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Steven L. Highlander

APPEAL BRIEF

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APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Appeal Brief is filed in response to the Office Action mailed on March 7, 2007. Appellants brief is due October 4, 2007, by virtue of the Notice of Appeal filed on June 1, 2007, and the enclosed Petition for Extension of Time (3 months) and payment of fees. Also included herewith is the fee for the brief. No other fees are believed due in connection with this filing; however, should appellants payments be missing or deficient, or should any fees be due, appellants authorize the Commissioner to debit Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/EPCL:010US/SLH.

I. Real Party In Interest

The real party in interest is the assignee, Athera Biotechnologies, AB, Stockholm, Sweden.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

A copy of the appealed claims is attached as Appendix A.

IV. Status of the Amendments

No "after final" amendments have been presented.

V. Summary of the Claimed Subject Matter

Claim 1, drawn to a method for diagnosing early cardiovascular disease, is supported in the specification as follows: (a) contacting a sample of body fluid with phosphocholine and/or a derivative thereof (Specification at paras. [0013-0014, 0016]), (b) assessing the presence and/or concentration of antibodies to phosphocholine and/or to said derivative in the sample by measuring antibodies bound to phosphocholine and/or derivative thereof (Specification at para. [0014]), and (c) diagnosing early cardiovascular disease based on the presence and/or concentration of said antibodies in the sample (Specification at para. [0004, 0013]).

VI. Grounds of Rejection to be Reviewed on Appeal

1. Claims 1-13 as allegedly obvious over the claims of U.S. Patent 6,780,605 (Exhibit 1) in view of Muzya *et al.* (Exhibit 2).
2. Claims 14 and 16-26 as allegedly obvious over the claims of U.S. Patent 6,780,605 (Exhibit 1) in view of Muzya *et al.* (Exhibit 2) and Baldo *et al.* (Exhibit 3).
3. Claims 1-3, 6-8, 11-14, 16, 17, 20-23 and 26 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4).
4. Claims 4, 9, 18 and 24 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4) and Barquinero *et al.* (Exhibit 5).
5. Claims 5, 10 19 and 25 as allegedly obvious over Muzya *et al.* (Exhibit 2) in view of Ostermann *et al.* (Exhibit 4) and Smal *et al.* (Exhibit 6).

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by

"substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejections for Alleged Obviousness-Type Double-Patenting

Claims 1-14 and 16-26 are rejected as allegedly obvious over the claims of the '605 patent, optionally taken with Muzya *et al.* Though traversing, appellants previously submitted a terminal disclaimer to address these rejections. However, the office action of March 3, 2007 indicates that the terminal disclaimer was not accepted, but no indication has been provided as to why the terminal disclaimer was deemed deficient. In the absence of such an explanation, appellants are at a loss to respond further other than to state that they remain willing to file the terminal disclaimer to obviate the rejection.

C. Rejections Under 35 U.S.C. §103

(i) Claims 1-3, 6-8, 11-14, 16, 17, 20-23 and 26 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.*

Claims 1-3, 6-8, 11-14, 16, 17, 20-23 and 26 are rejected as obvious over Muzya *et al.* in view of Ostermann *et al.* Muzya is cited as teaching that antibodies binding to PAF also bind to lyso-PAF and acyl analogs of PAF, and Ostermann is cited as teaching PAF quantification in serum and plasma as well as correlation/diagnosis with atherosclerosis. From this, the examiner concludes that "it would have been obvious ... to measure ***PAF concentrations in serum and plasma*** [of] patients with cardiovascular disease such as atherosclerosis ... because Ostermann *et al.* teach the critical role of PAF in myocardial infarction/atherosclerosis and its accuracy of correctly classifying subjects" (emphasis added). Once again, the examiner has (a) failed to recognize that she is advancing a rejection against a claim that is not pending, (b) failed to correctly interpret the Ostermann reference, and (c) failed to recognize that Muzya and

Ostermann are examining different things, and thus the conclusions drawn in one reference cannot be readily extrapolated to the other.

Claim 1 is drawn to "A method for diagnosing early cardiovascular disease comprising (a) contacting a sample of body fluid with phosphocholine and/or a derivative thereof, (b) assessing the presence and/or concentration of antibodies to phosphocholine and/or to said derivative in the sample by measuring antibodies bound to phosphocholine and/or derivative thereof, and (c) diagnosing early cardiovascular disease based on the presence and/or concentration of said antibodies in the sample." ***Thus, appellants first wish to make it clear that they are not examining PAF content in serum or plasma.*** Rather, they are examining ***antibodies to PAF or PAF derivatives***. Thus, the examiner's quoted statement above regarding the obviousness of measuring "PAF concentrations in serum and plasma" is completely off the mark for the simple reason that ***appellants don't measure PAF***. For this reason alone, the rejection should be reversed.

As discussed above, like the present invention Muzya examines anti-PAF antibodies. However, it has no further relevance with respect to present claim 1 as it only it addresses ***gynecologic disorders***. Thus, as a primary reference, this paper does little more than show that (a) anti-PAF antibodies do exist, and (b) that there ***might*** be some relationship between anti-phospholipid antibodies and anti-PAF antibodies. Yet as the examiner clearly recognizes, there is nothing in this paper to link anti-PAF antibodies to early cardiovascular disease (CVD).

Turning to Ostermann, the examiner has again apparently missed the key fact that this paper addresses an apparent correlation between the activity of a PAF acetylhydrolase activity and atherosclerosis, ***but says nothing about PAF levels, and nothing about anti-PAF antibody levels in subjects***. More troubling is the fact that the examiner actually argues that "Ostermann

et al. teach PAF quantification in serum and plasma ...," citing to the abstract and page 531, para. 2, of the paper. This false statement persists despite the fact that appellants' representative went over this very point during the interview held on November 15, 2006, and again in the previous response.

Thus, at the sake of belaboring the point, it is again pointed out that what is measured in Ostermann is PAF acetylhydrolase activity, and not PAF levels. This is readily observed by reading the same page 531, para. 2, cited by the examiner: "The degradation of PAF in serum was measured under standard conditions by a method similar to that described by BLANK *et al.* [4]. 50 µl serum dilution (1:19) were added to 0.5 ml of 11 µM ^{14}C-PAF ...." Thus, ***the PAF was added to the serum to assay for PAF aceytlhydrolase activity, and was not part of the serum obtained from the subjects***. Thus, Ostermann says ***nothing*** about PAF levels, and it's conclusions about atherosclerosis are thus moot.[1]

In sum, appellants submit that the cited references fail to render obvious the claimed invention for reasons already made of record – that Muzya deals with gynecologic orders, not CVD, and Ostermann deals with PAF acetylhydrolase levels, and not PAF levels.[2] For these reasons, reversal of the rejection is respectfully requested.

(ii) Claims 4, 9, 18 and 24 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.* and Barquinero *et al.*

Claims 4, 9, 18 and 24 are rejected as obvious over Muzya *et al.* in view of Ostermann *et al.* and Barquinero *et al.* The primary and secondary references are cited as above, and

[1] Fortunately, the examiner has not argued that increased PAF acetylhydrolase would increase PAF levels - indeed, if an increase in PAF acetylhydrolase activity exists with atherosclerosis, one would expect a ***drop*** in anti-PAF levels.

[2] Abstract statements that appellants cannot argue the references separately are of no help in clarifying the record. The deficiencies of the references by ***definition*** must be addressed individually.

Barquinero is merely cited for teaching ELISA's. However, as set forth in detail above, the primary and secondary references fail to establish the obviousness of using anti-PAF antibody levels to diagnose early CVD. Barquinero, whatever it might offer with regard to ELISA formats, cannot rescue the deficiencies of the other references given that it is directed to examining autoimmune disease, and not CVD. Thus, for the reasons given above, the rejection is improper and should be reversed as well.

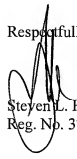
(iii) Claims 5, 10 19 and 25 as allegedly obvious over Muzya *et al.* in view of Ostermann *et al.* and Smal *et al.*

Claims 5, 10, 19 and 25 are rejected as obvious over Muzya *et al.* in view of Ostermann *et al.* and Smal *et al.* The primary and secondary references are cited as above, and Smal is merely cited for teaching radioimmunoassay's. However, as set forth in detail above, the primary and secondary references fail to establish the obviousness of using anti-PAF antibody levels to diagnose early CVD. Smal, whatever it might offer with regard to radioimmunoassay formats, cannot rescue the deficiencies of the other references given that it is directed to examining autoimmune disease, and not CVD. Thus, for the reasons given above, the rejection is improper and should be reversed as well.

D. Conclusion

In light of the foregoing, appellants respectfully submit that all pending claims are enabled and non-obvious over the cited art. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,



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VIII. APPENDIX A – APPEALED CLAIMS

1. A method for diagnosing early cardiovascular disease comprising (a) contacting a sample of body fluid with phosphocholine and/or a derivative thereof, (b) assessing the presence and/or concentration of antibodies to phosphocholine and/or to said derivative in the sample by measuring antibodies bound to phosphocholine and/or derivative thereof, and (c) diagnosing early cardiovascular disease based on the presence and/or concentration of said antibodies in the sample.

2. The method of claim 1, wherein said early cardiovascular disease comprises atherosclerosis, hypertension or thrombosis.

3. The method of claim 2, wherein measuring comprises an immunoassay.

4. The method of claim 2, wherein measuring comprises an enzyme linked immunosorbent assay.

5. The method of claim 2, wherein measuring comprises a radioimmunoassay.

6. The method of claim 2, wherein said body fluid is serum prepared from a blood sample.

7. The method of claim 2, wherein said body fluid is plasma prepared from a blood sample.

8. The method of claim 1, wherein measuring comprises an immunoassay.

9. The method of claim 1, wherein measuring comprises an enzyme linked immunosorbent assay.

10. The method of claim 1, wherein measuring comprises a radioimmunoassay.

11. The method of claim 1, wherein said body fluid is serum prepared from a blood sample.

12. The method of claim 1, wherein said body fluid is plasma prepared from a blood sample.

13. The method of claim 1, wherein said body fluid is a human blood sample or fraction

thereof, and said measuring comprises an immunoassay.

14. The method of claim 2, wherein said derivative is lysophosphatidylcholine.

16. The method of claim 1, wherein said derivative is lysophosphatidylcholine.

17. The method of claim 3, wherein said derivative is lysophosphatidylcholine.

18. The method of claim 4, wherein said derivative is lysophosphatidylcholine.

19. The method of claim 5, wherein said derivative is lysophosphatidylcholine.

20. The method of claim 6, wherein said derivative is lysophosphatidylcholine.

21. The method of claim 1, wherein said body fluid is contacted with phosphochline.

22. The method of claim 2, wherein said body fluid is contacted with phosphochline.

23. The method of claim 3, wherein said body fluid is contacted with phosphochline.

24. The method of claim 4, wherein said body fluid is contacted with phosphochline.

25. The method of claim 5, wherein said body fluid is contacted with phosphochline.

26. The method of claim 6, wherein said body fluid is contacted with phosphochline.

IX. APPENDIX B – EVIDENCE CITED

Exhibit 1 – U.S. Patent 6,780,605

Exhibit 2 – Muzya *et al.*

Exhibit 3 – Baldo *et al.*

Exhibit 4 – Ostermann *et al.*

Exhibit 5 – Barquinero *et al.*

Exhibit 6 – Smal *et al.*

X. APPENDIX C – RELATED PROCEEDINGS

None

EXHIBIT 1

US006780605B1

(12) **United States Patent**
Frostegård

(10) **Patent No.: US 6,780,605 B1**
(45) **Date of Patent: Aug. 24, 2004**

(54) **METHOD OF DIAGNOSING CARDIOVASCULAR DISEASE AND EARLY ATHEROSCLEROSIS**

(75) Inventor: **Johan Frostegård**, Nacka (SE)

(73) Assignee: **Athera Biotechnologies AB**, Stockholm (SE)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/720,967**

(22) PCT Filed: **Jul. 2, 1999**

(86) PCT No.: **PCT/SE99/01208**

§ 371 (c)(1),
(2), (4) Date: **Apr. 6, 2001**

(87) PCT Pub. No.: **WO00/02046**

PCT Pub. Date: **Jan. 13, 2000**

(30) **Foreign Application Priority Data**

Jul. 3, 1998 (SE) 9802402

(51) **Int. Cl.**[7] **G01N 33/567**; G01N 33/53

(52) **U.S. Cl.** **435/7.21**; 436/501; 436/512; 436/518; 435/7.1; 435/7.94; 435/7.93; 435/287.1; 435/7.9

(58) **Field of Search** 436/86, 89, 155, 436/161, 174, 811, 501, 512, 518; 435/5, 6, 7.1, 7.21, 7.94, 7.93, 7.9, 287.1, 288, 289.1, 291, 817

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,731,208 A 3/1998 Heinecke 436/86

OTHER PUBLICATIONS

Barquinero et al., "Antibodies against platelet activating factor in patients with antiphospholipid antibodies." Lupus, vol. 3, 1994.*

Ostermann et al., The degradation of platelet activating factor in serum and its discriminative value in atherosclerotic patients. Throbosis Research, 52, 1988, pp. 529–540.*

Karasawa et al. "Radioimmunoassay for platelet activating factor." Lipids, vol. 26, No. 12, 1991, pp. 1126–1139.*

Baldo et al. "A specific, sensitive, and high capacity immunoassay for PAF.", Lipids, vol. 26, No. 12, 1991, pp. 1136–1139.*

Lupus, vol. 3, 1994, Jordi Barquinero et al, "Antibodies Against Platelet–Activating Factor in Patients with Antiphospholipid Antibodies", p. 55—p. 58

Dialog Information Services, File 154, MEDLINE, Dialog accession No. 08672149, Medline accession No. 96350619, Hirashima Y et al: "Platelet–activating factor (PAF) and the formation of chronic subdural haematoma. Measurement of plasma PAF levels and anti–PAF immunoflobulin iiters"; & Acta Neurochir, 1995, 137 (1–2) p 15–8 abstract only.

Harris et al, Evaluation of the Anti–Cardiolipin Antibody Test: Report of an International Workshop held Apr. 4, 1986:, Clin. Exp Immunol, 1987 Apr; 68(1):215–222.

Abstract: Harris et al, Evaluation of the Anti–Cardiolipin Antibody Test: Report of an International Workshop held Apr. 4, 1986:, Clin. Exp Immunol, 1987 Apr;68(1):215–22.

Lemne et al, "Carotid Intima–Media Thickness and Plaque Borderline Hypertension" STROKE, 261(1); pp. 34–39; 1995.

Frostegard et al, "Association of Serum Antibodies to Heat–Shock Protein 65 With Borderline Hypertension", Hypertension, 29(1), pp. 40–44, 1997.

Frostegard et al, "Platelet–Activating Factor and Oxidized LDL Induce Immune Activation by a Common Mechanism", Arteriosclerosis, 17(5), pp 963–968, 1997.

* cited by examiner

Primary Examiner—Christopher L. Chin
Assistant Examiner—Lisa V. Cook
(74) *Attorney, Agent, or Firm*—Browdy and Neimark, P.L.L.C.

(57) ABSTRACT

The present invention provides a method of diagnosing cardiovascular disease and increased risk of early atherosclerosis in a human wherein the presence and/or concentration of antibodies to platelet activating factor (PAF) in a sample of body fluid of said human is assessed. The present invention further provides a kit carrying out said method.

12 Claims, No Drawings

METHOD OF DIAGNOSING CARDIOVASCULAR DISEASE AND EARLY ATHEROSCLEROSIS

The present application is the national stage under 35 U.S.C. 371 of PCT/SE99/01208, filed Jul. 2, 1999, itself based on U.S. application No. 60/091,741 filed Jul. 6, 1998, and claiming priority from Swedish application 9802409-9 filed Jul. 3, 1998.

FIELDS OF THE INVENTION

The present invention relates generally to methods for identifying patients who have cardiovascular disease and increased risk of developing atherosclerosis. More particularly, the invention relates to the detection of IgG antibodies to platelet activating factor (PAF) in body fluids of patients. The present inventors have shown that elevated concentrations of antibodies to PAF in body fluids is correlated to borderline hypertension and metabolic syndrome, i.e. early cardiovascular disease, which is connected to increased risk of developing early atherosclerosis.

BACKGROUND OF THE INVENTION

The morbidity and mortality associated with cardiovascular diseases and atherosclerosis in developed countries is higher than that associated with any other disorder. Hypertension is, together with hyperlipidemia, the most prominent risk factor for atherosclerosis. Individuals with borderline hypertension are an example of early cardiovascular disease in general, with endothelial dysfunction and increased risk of atherosclerotic disease, in apparently healthy individuals. Early atherosclerosis manifests itself in the form of cholesterol depositions in the arterial wall. During recent years, it has been convincingly shown that the atherosclerotic process is a chronic inflammation, characterized by presence of activated T cells and monocytes/macrophages. Many of these macrophages have developed into cholesterol-filled foam cells. The deposition is slow and starts at an early age. Clinical symptoms may take years to manifest themselves and are very serious; they include coronary heart disease and stroke. Generally, the disease process will have begun long before these clinical manifestations appear. There are available a number of genetic analysis screening for patients with pre-deposition for atherosclerosis. But it is desirable to have available a diagnostic technique which provides an early warning of the onset of the deposition. The importance of early detection is stressed by the fact that an effective long-term treatment is possible. The present techniques for diagnosing atherosclerosis depend on measuring cholesterol or triglycerid levels in serum or detection of atheromatous lesions, but by the time of detection, the most effective time for treatment has been passed. U.S. Pat. No. 5,731,208 discloses a screening test for atherosclerosis comprising determining the concentration of p-hydroxyphenylaldehyde-lysine in serum or plasma.

The present inventors have found that elevated concentrations of IgG antibodies to platelet activating factor (PAF) in patients are an indicator of cardiovascular diseases which is often accompanied by early atherosclerosis. More specifically, antibodies to PAF (aPAF) are associated with early vascular disease in the form of both borderline hypertension and the metabolic syndrome, both of which are strong risk factors for later stages of atherosclerosis, which give rise to clinical symptoms.

These results demonstrate that antibodies against PAF represent a novel category of anti-phospholipid antibodies

(aPL), which are sensitive to early vascular dysfunction and disease, especially early atherosclerosis and hypertension.

aPL in general, especially against cardiolipin have been shown to predict risk of cardiovascular disease, also in autoimmune diseases like systemic lupus erythematosus (SLE) and our data thus indicate that antibodies against PAF is a novel category of aPL, with a potential as a marker also in other autoimmune conditions in addition to cardiovascular disease and atherosclerosis in general. aPL have been related to both arterial and venous thrombosis, and also to spontaneous abortion. These data indicate that antibodies to PAF were much more strongly associated with spontaneous abortion than aPL, and furthermore, that antibodies to PAF was a novel marker for disease activity in SLE.

Antibodies to PAF are therefore relevant also in these other autoimmune vascular-related diseases.

Also antibodies to PAF-like lipids are relevant in this context, one being lysophosphatidylcholine, where the results indicate a comparable profile as the one obtained by PAF antibodies.

Accordingly, it is a principal object of the present invention to provide a diagnostic method or screening test for early atherosclerosis or cardiovascular changes related to early atherosclerosis. It is yet an other object of the invention to provide a kit for assaying the concentrations of aPAF for diagnosing early atherosclerosis or cardiovascular changes.

"Early atherosclerosis" as used herein refers to the very first stage of atherosclerosis, before the clinical onset of symptoms. "Early cardiovascular disease" as used herein refers to the first stages of cardiovascular disease, as in borderline hypertension and the metabolic syndrome, when atherosclerosis is yet not easy to detect by other methods and has not given rise to disease.

Platelet activating factor (PAF) is a phospholipid inflammatory mediator that is synthesized by a variety of cells, including monocytes and endothelial cells. During oxidation of LDL, PAF-like lipids are produced. PAF may therefore be of importance in pathological processes in the vascular wall like atherosclerosis and hypertension. In a previous report, the existence of antibodies to PAF (aPAF) were described in individuals with phospholipid antibody syndrome (Barquinero et al., 1994.), but nothing has been reported about possible clinical implications of these antibodies and a putative role in cardiovascular disease.

DISCLOSURE OF THE INVENTION

As mentioned above, we have surprisingly shown that concentration of antibodies to PAF (APAF) is an effective indicator of early cardiovascular disease. We have found that antibodies to this particular antigen develop in patients well before the clinical onset of atherosclerosis.

In our study we found that concentration of APAF was 49.3% higher in borderline hypertension men than in normotensive men the. When defining APAF concentrations above mean concentration of control plus two standard deviations (i.e. 0.144+(2×0.109)=0.362 OD405) as positive, 15 men out of 73 were positive in the borderline hypertension group whereas only 3 men out of 73 were positive in the normotensive group. Antibodies to PAF as a marker for early atherosclerosis may be combined with additional and alternative markers for early atherosclerosis to improve the accuracy of the diagnosis, such as determining the concentrations of cholesterol, blood lipids or p-hydroxyphenylaldehyde-lysine.

Antibodies against PAF (aPAF) may be determined using any of the methods and techniques conventional in the art for

such determination. Conveniently, such a method may comprise immunoassay e.g. ELISA or RIA. The immunoassay will conveniently use an antigen (PAF) in immobilized form, e.g. on microtitre plates, membranes or beads, to isolate the target aPAF. In a sandwich assay, the bound antigen may be labelled using additional soluble antibody, which may be monoclonal or polyclonal and which may either carry a label or, more conveniently, may itself be labelled subsequently by reaction with a secondary antibody carrying a label. Suitable labels include radionucleides, fluorescent substances, and enzymes.

Alternatively, a competitive binding assay may be used. Conveniently, the components needed to perform the immunoassay will be supplied in kit form. Such a kit would comprise:

a) an antigen capable of binding to aPAF and, optionally;

a labelled sample of antigen to aPAF or a fragment thereof;

said antigen (a) in non-immobilised form;

a labelled secondary antibody specific to said antigen (c).

The body fluid on which the determination is performed may be any body fluid in which APAF may be located, but conveniently will be or serum or plasma. In some cases it may be convenient to extract the antibodies, or otherwise treat the sample prior to determination.

The invention will now be described in greater detail by reference to the following non-limiting examples:

EXAMPLE 1

Determination of concentration of PAF antibodies of early atherosclerosis patients and of normal patients.

In order to investigate the role of aPAF in borderline hypertension (BHT) and early atherosclerosis, we studied a group of 146 middle aged men, where borderline hypertension were compared with age-matched controls. We here report that serum aPAF titers are enhanced in patients with borderline hypertension and metabolic syndrome.

Patients were recruited from a population screening program as previously described (Lemne et al 1995). BHT was defined as diastolic blood pressure (DBP) of 85 to 94 mmHg, and the screening identified 81 men who remained within the range for borderline hypertension during repeated measurements over a three year period. From the same population 80 age matched controls were recruited, whose blood pressure was measured on two occasions a few weeks apart, and was <80 mmHg on both occasions.

Of the 81 men with BHT and the 80 NT controls who agreed to participate, 73 in the BHT and 75 in the NT group completed all procedures of the present study.

None of the subjects had any other illnesses or were regularly using any drugs known to influence blood pressure, metabolic or inflammatory variables.

All subjects were investigated according to the same schedule. Both BHT and NT controls were investigated simultaneously when possible and no more than 4 weeks apart. Blood samples for analyses of metabolic and inflammatory variables were taken between 8 and 9:30 a.m., after 8 to 12 hours of fasting. All samples were drawn after 15 minutes of rest in the supine position.

An identical procedure was followed at each occasion during the entire recruitment period. All blood pressure measurements were performed with a mercury sphygmomanometer. The cuff was adjusted according to the circumference of the arm and placed at the level of the heart. Blood pressure was recorded as the mean of two measurements taken after 5 minutes rest in the supine position. Systolic and diastolic blood pressure measurements were defined according to Korotkoff I and V. The same specially trained nurse performed the measurements on all occasions.

The right and left carotid arteries were examined with a duplex scanner (Acuson 128XP/5, Mountain View, Calif., USA) using a 7.0 MHz linear array transducer. The subjects were investigated in the supine position and intima-media (I-M) thickness was determined in the far wall as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia's echo. Plaque was defined as a localized I-M thickening with a thickness>1 mm and a 100% increase in thickness compared with normal, adjacent wall segments. Plaque occurrence was scored as present or absent. Plaque was screened for in the common, internal and external carotid arteries on both sides, as described earlier (Lemne et al 1995).

All patients were weighed without other clothing than underwear, using the same scale (Delta 707, SECA, Germany). Length was measured with a special ruler, fixed to the wall. Waist circumference was measured at the level of the umbilicus and the hips were measured at the level of the greatest circumference. Body mass index (BMI) was subsequently calculated as weight in kilograms/(height in meters)^2

IgG antibodies to PAF were determined according to Example 2. PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) was obtained from Sigma, St Louis, USA.

Lipid and lipoprotein levels were determined by a combination of preparative ultracentrifugation followed by lipid analyses in the lipoprotein fractions as previously described (Lemne et al 1995).

Venous blood samples for determination of plasma insulin (Radio-Immuno Assay, Kabi Pharmacia, Sweden) were taken.

Serum immunoglobulins, IgG, IgM and IgA were determined as described (Frostegard et al, Hypertension 1997)

Variables were tested for skewness. For skewed variables non-parametric tests were used for comparisons between the groups (Mann-Whitney U-test), whereas Student's t-test was used for normally distributed variables. Spearman rank correlation coefficients were calculated to estimate interrelations between antibody levels, metabolic variables and blood pressure levels. The significance level was put at $p<0.05$. Values in the text are given as mean i standard deviation (SD) as indicated.

Results

Basic characteristics of the two study groups are presented in Table 1. The mean blood pressure level in the NT group was 125/75 (±11/±5) mmHg as compared to 141/89 (±10/±2) mmHg in the BHT group. The two groups were well-matched for age. The BHT men had a significantly altered metabolic profile with fasting hyperinsulinaemia and dyslipoproteinaemia, as previously presented (Table 1). In the BHT group 26% of the subjects had plaque on one or both sides while and the corresponding figure for the NT group was 16% (19 vs 10 subjects, n.s.).

In the material as a whole, the APAF levels were significantly higher in the BHT group, compared with the NT group (Table II). There was no difference in alysoPAF levels between the BHT and NT group.

There were no significant differences in APAF levels between individuals with plaque (n=29) compared to individuals without (n=117); data not shown).

If values above 2SD in the control group were defined as positive, 21% in the BHT group and 4% in the NT group had increased APAF levels. Age did not correlate with antibody levels (data not shown).

To exclude the possibility that differences in antibody levels simply reflected enhanced total antibody levels total IgG was determined. There was no difference between the BHT group and controls (data not shown).

In the material as a whole, and the two groups separately there were no significant correlations between APAF and BMI, blood pressure levels, or smoking (data not shown).

However, individuals with the metabolic syndrome (defined as having at least two of the following three conditions: BMI>27 kg/m^2, insulin levels above the 90th percentile of the normal population, dyslipoproteineamia) had higher APAF levels than those without (0.222±0.167 versus 0.169±0.106; p=0.0009).

Taken together, individuals with early cardiovascular disease, as in borderline hypertension, had 5 times higher risk of being positive for aPAF than those without.

TABLE I

	NT (n = 73)	BHT (n = 73)	p
Waist-hip ratio	0.90 (±0.05)	0.92 (±0.05)	0.022
Current smokers, %	37	32	
Cholesterol (mmol/l)			
Plasma	5.5 (±1.0)	5.5 (±0.9)	
HDL	1.27 (±0.27)	1.16 (±0.28)	0.016
Triglycerides (mmol/l)			
Plasma	1.34 (±0.80)	1.57 (±0.77)	0.015
VLDL	0.85 (±0.69)	1.0 (±0.68)	0.029
Insulin (mU/l)	14.2 (±4.5)	17.4 (±5.7)	0.0004

Values are given as mean ±SD. Group differences were determined by Student's t-test or Mann-Whitney's U-test (skewed variables). HDL=high density lipoprotein, VLDL= very low density lipoprotein.

TABLE II

Antibody levels to PAF in subjects with or without BHT or metabolic syndrome.

	NT (n = 73)	BHT (n = 73)	p
aPAF, OD405	0.144 ± 0.109	0.215 ± 0.130	p = 0.0007

Values are given as mean ±SD. Group differences were determined by Student's t-test. aPAF=antibody levels to platelet activating factor.

EXAMPLE 2

Method of determining the amount of antibodies to PAF (aPAF) in a serum sample.

IgG antibodies to PAF and lysoPAF were determined by an enzyme-linked immunsorbent assay (ELISA) essentially as described when phospholipid antibodies including cardiolipin are analysed (Harris 1986). Titertek® 96-well polyvinylchloride microplates (Flow Laboratories, Costa Mesa, Calif. USA) were coated with 50 μl/well of 50 μg/ml PAF dissolved in ethanol and allowed to dry overnight at 4° C. Blocking was accomplished with 20% ABS-PBS for two hours. 50 μl of serum samples, diluted 1:50 in 20% ABS-PBS were added to each well. Control assay were performed in the absence of PAF.

After 3 washings with PBS the plates were incubated with 50 μl/ml of alkaline phosphatase-conjugated goat anti-human IgG (Sigma A-3 150) diluted 1:9000 with PBS at 37° C. for 2 hours. After 3 washings, 100 μl of substrate (phosphatase substrate tablets, Sigma 104; 5 mg in 5 ml diethanolamine buffer, pH 9.8) was added. The plates were incubated in room temperature for 30 minutes and read in an ELISA Multiskan Plus spectrophotometer at 405 nm. Each determination was done in triplicate. The coefficient of variation between triplicate test was less than 5%.

REFERENCES CITED

Lemne C, Jogestrand T, de Faire U. Carotid intima-media thickness and plaque in borderline hypertension. Stroke 1995: 26;34–39.

Frostegard J, Lemne C, Andetsson B, Kiessling R and de Faire, U. Association of serum antibodies to heat shock protein 65 with borderline hypertension. Hypertension 1997:29:40–44.

Frostegard J, Huang Y H, Rönnelid J and Schäfer-Elinder L. PAF and oxidized LDL induce immune activation by a common mechanism. Arteriosclerosis, Thrombosis and Vasc Biol. 1997; 17: 963–968.

Barquinero J, Ordi-Ros J, Selva A, Perez-Peman P, Vilardell M, Khamashta M. Antibodies against platelet-activating factor in patients with antiphospholipid antibodies. Lupus 1994; 3:55–58.

Harris E N, Gharavi A E, Patel S P, Hughes G R V. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held Apr. 1, 1986. Clin Exp Immunol 1986;68:215–222.

U.S. Pat. No. 5,731,208

What is claimed is:

1. A method for diagnosing cardiovascular disease, comprising

providing contact between a sample of body fluid and an antigen capable of binding to an antibody to platelet activating factor (PAF),

assessing the presences and/or concentration of antibodies to in the sample of body fluid, and

evaluating said presences and/or concentration of antibodies to in the sample of body fluid as an indicator of cardiovascular disease.

2. The method of claim **1**, wherein said diagnosis of a cardiovascular disease comprises a diagnosis of early atherosclerosis, hypertension or thrombosis.

3. The method of claim **2** comprising measuring said antibodies to PAF by immunoassay.

4. The method of claim **2** comprising measuring said antibodies to PAF by an enzyme linked immunosorbent assay.

5. The method of claim **2** comprising measuring said antibodies to PAF by radioimmunoassay.

6. The method of claim **2** comprising measuring the concentration of said antibodies to PAF in serum prepared from a blood sample.

7. The method of claim **2** comprising measuring the concentration of said antibodies to PAF in plasma prepared from a blood sample.

8. The method of claim **1** comprising measuring said antibodies to PAF by immunoassay.

9. The method of claim **1** comprising measuring said antibodies to PAF by an enzyme linked immunosorbent assay.

10. The method of claim **1** comprising measuring the concentration of said antibodies in serum prepared from a blood sample.

11. The method of claim **1** comprising measuring the concentration of said antibodies to PAF in plasma prepared from a blood sample.

12. The method of claim **1** wherein said sample of body fluid is a human blood sample or fraction thereof, and said measurement is by immunoassay.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,780,605 B1
DATED : August 24, 2004
INVENTOR(S) : Johan Frostegård

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 6,
Lines 33 and 35, after "to", insert -- PAF --.
After line 65, insert new claim 13 as follows:
13. The method of claim 1 comprising measuring said antobodies to PAF by radioimmunoassay.

Signed and Sealed this

Twenty-first Day of December, 2004

JON W. DUDAS
Director of the United States Patent and Trademark Office

EXHIBIT 2

UDC 618.3-092:812.087.1]-078-33

Reaction of antiphosphatidylcholine antibodies with thrombocyte-activating phospholipid factor and its structural cellular analogues

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Scientific Centre for Obstetrics, Gynaecology and Perinatology,
Russian Academy of Medical Sciences, Moscow

The high proportion of antiphospholipid antibodies to membrane phospholipids is often associated with obstetrical pathology; this includes recurrent foetal loss, intrauterine growth retardation, hypertension in pregnancy, preeclampsia and thromboembolic complications [15]. In such cases, the blood serum of patients contains antibodies to the main cellular phospholipids such as cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, sphyngomyelin and phosphatidylcholine [10]. Study of the specific mechanisms of antiphospholipid antibody participation in the development of the pathology of pregnancy continues [14, 15]. It has been suggested that the rise observed in pregnancy in the level of phospholipids in the blood due to an increase in their anabolism, the deportation of syncytiotrophoblast microvilli and the release of phospholipid vesicles by the placenta stimulate the production of antiphospholipid antibodies [10].

Antiphospholipid antibodies (aPL) are apparently reactive not only with 'excess' phospholipids in the blood serum but also with lipoproteins and cells containing phospholipid antigen determinants on the cell surface [14].

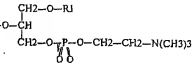
It is known that given the correct stimulus virtually all cells in mammals will release a universal phospholipid bioregulator, platelet-activating factor (PAF) [3] and its choline-containing cellular analogues - acyl and plasmalogen [4]. Phospholipid PAF is involved in the regulation of the blood clotting system, the cardio-vascular system and the immune system and is a mediator of inflammation with a range of etiologies, of allergic reactions and many other pathophysiological processes. PAF plays a major role in mammalian reproduction, with an effect on virtually all stages of the reproductive process, from functional development of the gametes, fertilisation and

embryo implantation to childbirth [6]. Since PAF is in terms of its chemical structure a choline-containing phospholipid, it may be expected that aPL antibodies and aPC antibodies in particular would be reactive with PAF and its structural analogues, with an effect on their biological activity.

The aim of this study was to investigate the reaction of blood serum containing antiphosphatidylcholine antibodies with PAF and its structural analogues.

Research method

Preparations of highly purified phospholipids of the following structure were used in the research project.



1) phosphatidylcholine: R1 is C16:0 and C18:0 fatty acid residues; R2 is C18:1 and C18:2 fatty acid residues
2) lysophosphatidylcholine: R1 is C16:0 and C18.0 fatty acid residues; R2 is H
3) phospholipid PAF: R1 is (CH2)15,17CH3; R2 is CH3CO;
4) PAF lysoderivative (lyso-PAF): R1 is (CH2)15, 17CH3; R2 is H
5) acyl analogue of PAF (1-acyl-PAF): R1 is C16:0 and C18.0 fatty acid residues; R2 is CH3CO.

Phosphatidylcholine is separated from egg yolks by the usual method [1]. Lysophosphatidylcholine (1-acyllysoglycero-3-phosphocholine) was obtained by cleaving egg phosphatidylcholine with phospholipase A2 and purified by column chromatography in L 100/160 μm silica gel [1]. 1-0-alkyllyso-*sn*-glycero-3-phosphocholine (lyso-PAF) was obtained by hydrogenating bovine heart choline plasmalogens, followed by alkaline hydrolysis as described earlier [1]. Phospholipid PAF was obtained by acetylating 1-0-alkyllyso-sn-glycero-3-phosphocholine with acetic anhydride in a chloroform medium and purifying by column chromatography in L 100/160 μm silica gel [2]. The PAF acyl analogue (1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained by acetylating the lysophosphatidylcholine with acetic anhydride in a chloroform medium and purifying by column chromatography in L 100/160 μm silica gel [7].

Murine monoclonal antibodies to human immunoglobulins (IgM, IgG), labelled horseradish peroxidase (Institute of Virus Preparations, Moscow), gelatine (N.A. Semashko Moskhimpharmpreparat), o-phenylendiamine (Sigma), hydrogen peroxide (Reakhim) and polystyrene microplates manufactured by GosNIIMedpolimer (Moscow) were used for the enzyme immunoassay (EIA). Blood serum samples taken in the Scientific Centre of Obstetrics, Gynaecology and perinatology of the Russian Academy of Medical Sciences from patients with recurrent foetal loss, late toxicosis in pregnancy, history of perinatal foetal death, infertility and unsuccessful attempts at *in vitro* fertilisation and embryo transfer.

EIA was used to study the manner in which aPL antibodies bind with PAF and its structural analogues. Highly purified phospholipids (phosphatidylcholine, PAF, lyso-PAF, 1-acyl-PAF, lysophosphatidylcholine) were dissolved in a 50 μg/ml methanol concentration. The resultant phospholipid solutions were placed onto polystyrene microplates in quantities of 50 μm per well and incubated at 37°C for 18 ± 2 hrs. After each stage of the assay the plates were washed 4 times with 0.01 M phosphate buffer solution (pH 7.4 ± 0.2). After adsorption of the phospholipids the wells were treated with a 0.5% gelatine solution, 100 μm per well, at 20 ± 2 °C for 1.5 hr. A phosphate buffer solution containing 0.5% gelatine was used for cultivating the test samples of blood serum and conjugates. 75 μl assay samples of blood serum cultivated in a 1:50 proportion were inserted per well and incubated at 20 ± 2 °C in an agitator for 1.5 hr. Conjugates of murine monoclonal antibodies, with horseradish peroxidase, to human IgM and IgG, in 1:100000 and 1:50000 proportions respectively, were placed in the wells in amounts of 50 μl per well and incubated at 20 ± 2 °C in an agitator for 1 hr. After washing, a chromogen substrate solution containing o-phenylendiamine and hydrogen peroxide was added to the wells and the optical density (OD) was measured after 10 minutes at 492 nm using a Labsystems Multiscan MCC/340 photometer. The results of the assay were considered positive if the average OD of the assay sample was greater than the total of the average OD for the negative controls and two average mean square deviations.

Results and Discussion

To study the way antiphosphatidylcholine (aPC) antibodies bind with phospholipid PAF and its structural analogues, blood serum containing IgM, or IgM and IgG phosphatidylcholine antibodies was taken from patients presenting with obstetric and gynaecological pathologies. In

the case of the patient with late toxicity in pregnancy the IgG level was relatively higher than the IgM level, while in the other cases the IgM level was higher.

The EIA results indicated that serums containing IgM and IgG aPC antibodies react *in vitro* with the PAF and its analogues adsorbed onto the polystyrene plates. In addition, the linking of IgM antibodies with phosphatidylcholine was approximately 1.5 - 2 times higher than with PAF, lyso-PAF and 1-acyl-PAF adsorbed under the same conditions, and 3 times higher than with lysophosphatidylcholine (Table 1). No substantial differences were found in the degree of the reaction of aPC antibodies with PAF, lysoPAF and 1-acyl-PAF. The cross reaction typical for antiphospholipid antibodies had obviously occurred in this case.

Table 1

Level of IgM aPC antibodies in blood serum of patients with obstetric and gynaecological pathologies, and the binding with PAF and its structural analogues found from EIA

Patient group	Phospholipid tested				
	Phosphatidyl-choline	PAF	Lyso-PAF	1-acyl-PAF	Lyso-phosphatidyl-choline
Patients with death of infant in neonatal period	0.580 ± 0.035	0.759 ± 0.044	0.387 ± 0.023	0.386 ± 0.022	0.268 ± 0.015
Patients with foetal loss	0.320 ± 0.016	0.576 ± 0.034	0.243 ± 0.014	0.208 ± 0.012	0.145 ± 0.018
Patients with late toxicosis in pregnancy	0.400 ± 0.024	0.645 ± 0.065	0.293 ± 0.017	0.229 ± 0.013	0.378 ± 0.022
Patients with infertility	0.541 ± 0.031	0.727 ± 0.073	0.410 ± 0.024	0.356 ± 0.021	0.268 ± 0.016
Healthy fertile women	0.050 ± 0.003	0.126 ± 0.007	0.062 ± 0.004	0.051 ± 0.003	0.074 ± 0.004

Note: In Tables 1 and 2 the values given are for average OD at 492 nm ± σ

In the serum of patients with low levels of IgG aPC antibodies the differences in the way they bind with PAF and its analogues were slight (Table 2). However in the serum of the patient with late toxicosis in pregnancy a high level of IgG antibodies reactive with PAF and, significantly, to a lesser extent with its analogues was noted. It is not impossible that this patient had specific antibodies to PAF.

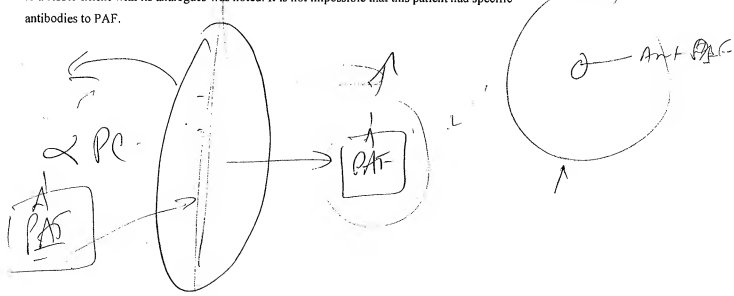


Table 2

Level of IgG aPC antibodies in blood serum of patients with obstetric and gynaecological pathologies, and the binding with PAF and its structural analogues found from EIA

Patient group	Phospholipid tested				
	Phosphatidyl-choline	PAF	Lyso-PAF	1-acyl-PAF	Lyso-phosphatidyl-choline
Patients with death of infant in neonatal period	0.189 ± 0.011	0.227 ± 0.013	0.141 ± 0.015	0.098 ± 0.006	0.086 ± 0.005
Patients with foetal loss	0.126 ± 0.008	0.250 ± 0.016	0.127 ± 0.009	0.089 ± 0.006	0.076 ± 0.008
Patients with late toxicosis in pregnancy	0.574 ± 0.034	1.011 ± 0.059	0.152 ± 0.010	0.097 ± 0.006	0.149 ± 0.009
Patients with infertility	0.085 ± 0.005	0.221 ± 0.014	0.114 ± 0.007	0.089 ± 0.006	0.050 ± 0.003
Healthy fertile women	0.061 ± 0.004	0.134 ± 0.008	0.064 ± 0.004	0.057 ± 0.004	0.050 ± 0.003

It is known that the antibodies to PAF may be evoked in rabbits after the introduction of PAF preparations containing C6:0 and C:12 alkyl residues, and PAF analogues (1-0-(ω-oxyalkyl)-2-acetyl-*sn*-glycero-3-phosphocholine, 1-0-(15'-carboxypentadecyl)-2-N, N-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine), covalently linked to methylated BSA [8, 11, 17, 18]. The identified antibodies to PAF were highly specific and were not reactive with lyso-PAF, PAF enantiomer, PAF methoxy analogue, lysophosphatidylcholine, phosphatidylcholine or PAF analogues containing propionic or butyric acid residues at the *sn*-2 position [8, 11, 17]. With the different molecular types of PAF containing C16:0, C18:0 and C18:1 alkyl residues at the *sn*-1 position, there were some small variations in the bonding of the antibodies, and the greatest bonding of antibodies was observed in C18:1 PAF [8]. These results indicate that the high specificity of antibodies to PAF depends on the recognition of the acetyl group at the *sn*-2 position and the trimethylammonium group of phosphocholine in the PAF molecule [17].

In contrast to the highly specific antibodies to PAF, aPC antibodies are not highly specific and are reactive with other phospholipids. It has been shown that antibodies to phosphatidcholine can be evoked in experimental animals by introducing erythrocytes, an emulsion of dipalmitoyl phosphatidylcholine in BSA or phosphatodylcholine liposomes, and they can also be produced by hybridoma technology [9, 12, 13, 16, 19]. aPC antibodies are also capable of binding with

lyso-phosphatidylcholine and sphyngomyelin [12], that is, they are capable of recognition of phosphocholine fragments of the polar part of phospholipids.

The results of this study show that IgM and IgG aPC antibodies in blood serum from patients with obstetric and gynaecological pathologies are capable of binding *in vitro* with PAF and its structural analogues which differ from PAF in the type of bond at the *sn*-1 position: a simple ether bond in the case of PAF and an ester bond in the case of 1-acyl-PAF.

What are implications of this observable reaction of aPC antibodies with PAF and its analogues in the pathogenesis of antiphospholipid syndrome (APS)? Thrombosis of the vessels of the placenta is thought to be the main mechanism in the development of obstetric pathology, with one of the causes of its occurrence being the major role played by the reaction of aPL antibodies with endothelial cells and thrombocytes [14]. It has been shown that the binding of aPL antibodies with endothelial cells leads to a reduction in the synthesis of prostacyclin, while their reaction with thrombocytes initiates the activation of thrombocytes and subsequent increase in the synthesis of thromboxane A2 and the release of adenosine diphosphate (ADP) [15]. At the same time, due to the presence of anticardiolipin antibodies, the endothelial cells release PAF [6]. Thus the increased production by cells of proaggregating agents such as PAF, thromboxane A2 and ADP along with the reduction in the synthesis of prostacyclin can cause the formation of intravascular aggregates of thrombocytes.

It is known that, in the blood circulation, PAF, released by cells binds with albumin and plasma lipoproteins [2] while free PAF is cleaved by acetylhydrolase associated with low density lipoproteins [5]. aPC antibodies can, apparently, bind with PAF in the microenvironment of cells actively producing PAF. It can be suggested that the formation of a compound with an antibody can inhibit the cleavage of PAF by acetylhydrolase.

An another important implication of the reaction of aPC antibodies with PAF may be the disturbance of the process of fertilisation of oocytes by spermatozoids. It is known that PAF stimulates spermatozoid motility, the acrosome reaction, and the process of fertilisation and implantation of the embryo [6]. Apparently aPC antibodies can significantly disturb these processes by removing PAF from the interaction of cells in the reproductive system. It is possible that unsuccessful attempts at *in vitro* fertilisation may be associated with a disturbance of the process of fertilisation and implantation of the embryo as a result of the binding of PAF

with aPC antibodies. It is therefore possible to suggest new links between APS and disturbances of the fertilisation processes in humans.

CONCLUSIONS

1. Antiphosphatidylcholine antibodies in the blood serum of patients with an obstetric and gynaecological pathology bind *in vitro* with phospholipid PAF, PAF lysine derivatives and PAF acyl analogues.

2. Antiphosphatidylcholine antibodies bound with PAF and its structural cell analogues are likely to be associated with the presence of phosphocholine fragments in the structure of certain phosphoglycerides.

LITERATURE

1. *Bergelson L.D., Dyatlovitskaya E.V., Molotkovskii Yu.G.* Preparativnaya biokhimiya lipidov [Preparative biochemistry of lipids]. M., 1981.
2. *Kulikov V.I., Muzya G.I., Bergel'son L.D.* // Biokhimiya. 1984. Vol. 49, N. 9. P. 1449-1455.
3. *Kulikov V.I., Muzya G.I.* // Ditto. 1992. Vol. 57, N. 6. P. 809-816.
4. *Kulikov V.I., Muzya G.I.* // Ditto. 1996. Vol. 61, N. 3. P. 387-403.
5. *Muzya G.I., Kulikov V.I.* // Ditto. 1991. Vol. 56, N. 6. P. 1140-1144.
6. *Muzya G.I., Kulikov V.I., Van'ko L.V., Sukhikh G. T.* // Akush i gin. 1996. N. 6. P. 12-16.
7. *Orlov S.M., Kulikov V.I., Pol'ner A.A., Bergel'son L.D.* // Biokhimiya. 1985. Vol. 50, N. 4. P. 680-685.
8. *Baldo B.A., Smal M.A., McCaskill A.C.* // Lipids. 1991. Vol.26, N 12. P. 1136-1139.
9. *Cox K.O., Hardy S.J.* // Immunology. 1985. Vol. 55. P. 263.
10. *Kajlno T.* // Amer. J. Reprod. Immunol. 1991. Vol. 25. P. 28-34.
11. *Karasawa K., Satoh N., Hongo T.* et al. // Lipids. 1991. Vol. 26, N 12. P. 1126-1129.
12. *Niedleck B., Kuck U., Gardemin H.* // Immunochemistry. 1978. Vol. 15. P. 471-475.
13. *Poncet P., Kocher H.P., Pages J.* et al. // Mol. Immunol. 1985. Vol. 22. P. 541-551.
14. *Rote N.S., Walter A., Lyden T.W.* // Amer. J. Reprod. Immunol. 1992. Vol. 28. P. 31-37.
15. *Rote N.S.* // Immunology of Reproduction / Ed. R.K. Naz. New York, 1993. Ch. 8. P. 147-167.
16. *Schuster B.G., Neidig M., Alving B.M., Alving C.R.* // J. Immunol. 1979. Vol. 122. P. 900-904
17. *Smal M.A., Baldo B.A., Redmond J.W.* // Mol. Immunol. 1989. Vol. 26, N 8. P. 711-719.
18. *Smal M.A., Baldo B.A., McCaskill A.* // J. Immunol. Meth. 1990. Vol. 128. P. 183-188.
19. *Wassey N.M., Roerdink F., Swartz G.M.* et al.// Mol. Immunol. 1984. Vol. 21. P. 863-868.

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Article received: 17 January 1997

Trans notes:
1. *thrombocyte activating phospholipid factor*: The literal translation has been used in the title, since it is a title. Elsewhere in the text the more usual English platelet activating factor, PAF, has been used.

2. *structural cellular analogues*: this Russian term has been shortened to 'structural analogues' throughout the translation.

3. *Patient:* in this text, the Russian uses the word 'female patient'.

4. *Phosphatidylinositol*: This term has been used to translate the Russian 'phosphatidylinosite'.

5. *late toxicosis in pregnancy*: the Russian term, 'OPG-gestoz', was introduced in 1987 for late toxicosis in pregnant women; the *O G P* stands for oedema, proteinuria and hypertension.

EXHIBIT 3

PCT

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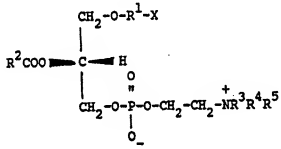
(54) Title: ANTIGENIC ANALOGUES OF PLATELET ACTIVATING FACTOR (PAF)



(I)

(57) Abstract

Antigens for the production of antibodies to Platelet Activating Factor (PAF). The antigens are PAF analogues of formula (I), wherein X comprises a high molecular weight group, R^1 is a linking group and R^2 to R^5 are selected from C_1 to C_6 alkyl. Other aspects of the invention include PAF-antibodies produced using said antigens, labelled PAF analogues, intermediates for the preparation of PAF analogues and methods and a kit for the immunoassay of PAF.

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ANTIGENIC ANALOGUES OF PLATELET ACTIVATING FACTOR (PAF)

Technical Field

The present invention relates to novel antigens capable of producing antibodies to Platlet Activating Factor (PAF), novel PAF analogues labelled to enable quantitative assay, intermediates for the production of novel PAF antigens and methods for the preparation of said antigens, and methods of immunoassay of PAF in biological fluid using said labelled analogues and/or labelled PAF-antibodies.

Background

Platelet Activating Factor (PAF), 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine, represents a recently defined example of a class of biologically-active lipids active in the subnanomolar range and possessing a wide spectrum of pathophysiological effects. PAF promotes life-threatening anaphylactic reactions in animals and is suspected of mediating a range of allergic and inflammatory reactions in man. For example, PAF may be important in conditions such as asthma, adult respiratory distress syndrome and shock reactions. However, despite the increasing catalogue of conditions in which PAF maybe involved, greater insights into its role in health and disease are hampered because precise and specific methods

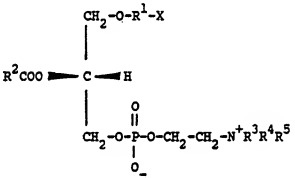
for its measurement are lacking. The capacity of PAF to aggregate platelets does not provide a suitable basis for strictly quantitative assay.

It would be desirable to develop an immunoassay for quantitative determination of PAF levels in blood serum. However, it has been found that PAF itself is insufficiently antigenic to produce the necessary PAF-antibodies needed for such an immunoassay.

Novel synthetic PAF analogues have now been found which are sufficiently antigenic to produce PAF-antibodies and a method suitable for the immunoassay of PAF levels in biological fluids has been developed.

The Invention

Accordingly the invention provides novel compounds of general formula (I) -



wherein:

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;

X is hydrogen; or

(2) R^4 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;

X is selected from:

(a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; and

(b) the group -A-B wherein A is a linking group selected from the groups $-NR^6-$, -COO-, -OCO-, $-CONR^6-$, $-NR^6CO-$, -NH-CS-NH- and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is selected from:

(i) monofunctional and polyfunctional protein peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000; and

(ii) a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl; and mixtures of the compound of formula (I) and its enantiomer.

In one embodiment the invention provides antigenic PAF analogues of general formula (I) wherein:

R^1 is a C_2 to C_{25} alkylene or alkenylene linking group;

X is the group -A-B wherein:

A is a linking group selected from $-NR^6-$, -COO-, -OCO-, $-CONR^6-$, $-NR^6CO-$ and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000 which are capable of eliciting an antigenic response; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

In the antigenic PAF analogues of the invention of general formula (I):

-Preferred R^1 include straight chain C_4 to C_{16} alkylene. More preferred R^1 include straight chain C_4 to C_8 alkylene. For convenience R^1 is often chosen from pentylene and hexylene.

-Preferred A include $-NR^6-$, -COO-, -OCO-, $-CONR^6-$ and R^6CO- and preferred R^6 include hydrogen and methyl. More preferred A include $-NR^6-$ and -OCO-.

-Preferred B include monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups of molecular weight at least 5000 and capable of eliciting an antigenic response. More preferred B include monofunctional and polyfunctional groups of molecular weight at least 10,000. Examples of suitable B include Bovine Serum Albumen (BSA), ovalbumen, Porcine Thyroglobulin (PTG), Bovine Thyroglobulin (BTG), keyhole

limpet haemocyanin, bacterial cell walls, synthetic polypeptides such as polylysine, poke weed mitagen (PWM), phytohaemoglutinin (PHA), muranyl dipeptidase and lipo-polysaccharides.

-Preferred R^2 to R^5 include C_1 to C_3 alkyl, and especially methyl.

In another embodiment the invention provides labelled PAF analogues of general formula (I) wherein:

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine; X is hydrogen; or

(2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group;

X is a group of formula -A-B wherein:

A is a linking group selected from $-NR^6-$, -COO-, -OCO-, $-CONR^6-$, $-NR^6CO-$, -NH-CS-NH-and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl;

B is a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

In the labelled PAF analogues of the invention of general formula I wherein X is hydrogen:

-Preferred R^1 include straight chain C_4 to C_{16} alkylene or alkenylene substituted by radioactive iodine.

-Preferred R^2 to R^5 are methyl.

In the labelled PAF analogues of the invention of general formula I wherein X is a group of formula -A-B:

-Preferred R^1 include straight chain C_4 to C_{16} alkylene, alkenylene or alkynylene. More preferred R^1 include straight chain C_4 to C_8 alkylene.

-Preferred A include $-NR^6-$, -COO-, -OCO-, $-CONR^6-$ and $-NR^6CO-$ and preferred R^6 include hydrogen and methyl. More preferred A include $-NR^6-$ and -OCO-.

-In this specification, "label" is used to mean conventional labels used in immunoassay procedures including : the radioactive isotope labelled groups based on ^{125}I-histamine, ^{125}I-tyramine, ^{125}I-tyrosine methyl ester and ^{125}I-Bolton Hunter Reagent; enzymic labels; and photometric labels. Specific examples emzymic labels include horseradish peroxidase, alkaline phosphatase, betagalactosidease and urease. Specific examples of photometric labels include fluorescent groups such as fluorescein and its derivatives, rhodamine and its derivatives, phycoerythrins, europium, "Texas Red", luminescent labels such as luminol and its derivatives, acridinium esters and umbelliferins.

-Preferred R^2 to R^5 are C_1 to C_3 alkyl, especially methyl.

In another embodiment the invention provides

compounds of general formula (I) which are useful as intermediates for the preparation of the antigenic PAF analogues of the invention wherein:

R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group; and

X is selected from the group consisting of formyl, carboxy, di(C_1 to C_6 alkoxy)methyl, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto.

In the intermediate compounds of the invention of general formula I:

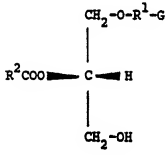
-Preferred R^1 include straight chain C_4 to C_{16} alkylene, alkenylene and alkynylene. More preferred R^1 include straight chain C_4 to C_8 alkylene.

-Preferred X include formyl, carboxy, dimethoxymethyl and hydroxy.

In another embodiment the invention provides a process for the preparation of compounds of general formula (I) which process comprises:

(a) reacting:

a compound of general formula (II)

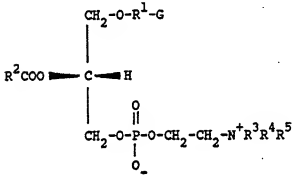


II

wherein R^1 and R^2 are as hereinbefore defined and G is selected from di(C_1 to C_6 alkoxy)methyl and groups which may be reacted, using conventional methods, to give a group selected from formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, amino, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto;

a phosphorylation agent; and

an N,N,N-tri(C_1 to C_6 alkyl)ethanolamine derivative to give a compound of general formula (III)



(b) reacting the product of (a) to convert group G as hereinbefore defined to a group selected from formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, amino, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto and to introduce the desired group X.

In a specific example of the process for the preparation of compounds of general formula (I):

(a) a compound of general formula (II), wherein G is dimethoxymethyl, R^1 is selected from C_4 to C_{16} alkylene, alkenylene and alkynylene and R^2 is methyl, is reacted with phosphorus oxychloride and choline tosylate to give a compound of formula (III), wherein G is dimethoxymethyl, R^1 is selected from C_4 to C_{16} alkylene, alkenylene and alkynylene, and R^2 to R^5 are methyl; and

(b) the product of (a) is reacted with acid to give a compound of formula (I) wherein X is formyl and R^1 to R^5 are as hereinbefore defined, which is reacted with a protein or synthetic peptide followed by reduction of the resulting imine to give a compound of general formula (I) wherein R^1 to R^5 are as hereinbefore defined and X is the group -A-B wherein A is the linking group $-NR^6-$ in which R^6 is hydrogen and B is a protein or synthetic peptide.

It will be recognized by those skilled in the art that in those antigenic PAF analogues of general formula I the group B may be monovalent or polyvalent such that a plurality of residues of general formula (I), typically between 1 and 500 and usually between 2 and 20, are attached to each group B. Therefore, in those antigenic PAF analogues of general formula I in which X is the group -A-B, if the residue of formula (I) is represented by Z then the invention includes antigenic PAF analogues of formula $(Z)_nB$ wherein n is an integer from 1 to 500.

It will also be recognized by those skilled in the art that certain of the PAF analogues of general formula (I) may be non-covalently bonded to or adsorbed onto a solid support. Accordingly in another embodiment the invention provides supported PAF analogues comprising PAF analogues of general formula (I) wherein:

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;
X is hydrogen; or

(2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;
X is selected from:

(a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl) amino, hydroxy and mercapto; and

(b) the group -A-B wherein A is a linking group selected from the groups $-NR^6-$, -COO-, -OCO-, $-CONR^6-$, $-NR^6CO-$, -NH-CS-NH- and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and B is a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl; non-covalently bonded to or adsorbed onto a solid support material.

Examples of solid support materials for said supported PAF analogues include proteins, synthetic polypeptides (eg polylysine) carbohydrates and carbohydrate derivatives [e.g. nitrocellulose, agaroses

such as "Sepharose" (Trade Mark), and lipopolysaccharides] and synthetic polymers such as, for example, polysulphones, polyamides (e.g. polyacrylamide, nylon 6, nylon 66, nylon 610) and polystyrene in the form of particles, balls or formed articles such as test--tubes, rods, tubes, fins, wells, beads, disks, slides, plates and micro-titre plates.

Although PAF itself has been found to be insufficiently antigenic to produce the PAF-antibodies required to develop an immunoassay for PAF, surprisingly it has been found that:

(a) PAF adsorbed onto or non-covalently bound to a monofunctional or polyfunctional protein, peptide, carbohydrate, lipid or a derivative thereof of molecular weight at least 2000 and capable of eliciting an antigenic response; and

(b) the antigenic PAF analogues of general formula (I); stimulate the production of antibodies which are antibodies to PAF. Accordingly in a further embodiment the invention provides antibodies to PAF and methods for their production. Such antibodies, hereinafter referred to as PAF-antibodies or anti-PAF, may be prepared by those techniques known in the art and conventionally involve introducing an antigenic PAF analogue of general formula (I) into an animal such as a rabbit, mouse, donkey, sheep, etc. to produce antibodies to the antigen and isolating and purifying the antibodies. The PAF-antibodies of the invention may be labelled with any of the conventional labels used in immunoassay

procedures. Such labels include, for example, radioactive labels, enzymic labels and photometric labels such as those hereinbefore described.

The PAF antibodies of the invention include both monoclonal antibodies and polyclonal antibodies and techniques known in the art may be utilized to prepare the required type of antibody. For example, monoclonal antibodies may be produced using the antigenic PAF analogues of general formula (I) of the invention by the techniques taught by G. Kohler and C. Milstein, Nature,256, 495-497 (1975).

The PAF analogues and PAF antibodies of the invention may be used to qualitatively and quantitatively analyse for the presence of PAF in biological fluids. Accordingly in a further embodiment the invention provides methods for the immunoassay of PAF in biological fluids using the PAF analogues and/or PAF-antibodies of the present invention.

In one method PAF or PAF analogue is immobilised on a solid support and reacted with labelled or unlabelled PAF-antibodies in the presence of known amounts of competing free PAF to generate a graph showing percent inhibition versus PAF concentration. If, unlabelled PAF antibody is used the antibody bound which binds to the first is detected by using a labelled second antibody (goat, donkey, sheep, etc.). Using this graph the amount of free PAF in biological fluids may be quantitatively measured.

In another method, unlabelled anti-PAF bound to a

solid support is reacted with a polyvalent antigenic PAF analogue of formula $(Z)_nB$ (e.g. PAF-polylysine). The resulting complex is then determined using labelled anti-PAF which binds to free PAF residues on the polyvalent antigenic PAF analogue.

In another method, unlabelled anti-PAF bound directly, either covalently or non-covalently, to a solid phase such as magnetized particles, plastic tubes, micro-titre plates,"Sepharose" (Trade Mark) particles, polyacrylamide particles, nylon or polystyrene balls, etc. is mixed in a competition assay with: (a) a known quantity of labelled PAF; and (b) known quantities of unlabelled PAF contained in standard solutions or PAF to be measured in an extract or biological fluid. The concentration of unlabelled PAF in the sample is then determined from a standard curve, for example from a logit/log standard plot.

In another method, the procedure above is used except that the anti-PAF is linked to the solid phase by a ligand such as an antibody, protein A, lectin or an enzyme, for example:

-solid phase/sheep (or some other species) anti-rabbit(or mouse etc.) immunoglobulin/rabbit (or mouse etc.) anti-PAF; and

-solid phase/protein A/rabbit (or mouse etc.) anti-PAF.

In another method, anti-PAF, labelled PAF and PAF to be measured are mixed and the free PAF and antibody-bound PAF are separated using dextran-coated charcoal or some other solid phase adsorbent such as hydroxyapatite etc. The concentration of unlabelled PAF in the sample being

measured is then determined from a standard curve.

In another method, anti-PAF/PAF complexes are precipitated with a second antibody or with a protein precipitating reagent such as ammonium sulphate. Again, concentrations of unlabelled PAF may be determined from a standard curve.

In a further embodiment the invention also provides a kit for the immunoassay of PAF in a biological fluid said kit comprising PAF-antibodies of the present invention.

In practice, it has been found that the PAF present in biological fluids such as blood serum is rapidly degraded by the enzyme PAF-acetylhydrolase which is also normally present in blood serum. Therefore, it is preferable to first deactivate the enzyme. Three methods have been published for the deactivation of the enzyme, namely use of 1N hydrochloric acid, use of diisopropylfluorophosphate, and use of phenylmethanesulphonyl fluoride, but these methods suffer the disadvantages of use of drastic conditions and/or toxic substances.

It has now been found that the addition of a detergent to the biological fluid sufficently deactivates the enzyme to enable PAF to be quantitatively determined. Therefore, in a further embodiment the invention provides a method of immunoassay of PAF in biological fluid which comprises diluting the fluid with an aqueous detergent solution, prior to subjecting the diluted fluid to an immunoassay. Preferably the detergent is a non-ionic detergent, such as those selected from the group

consisting of: polyalkylene glycols; alcohol, phenol and alkylphenol alkoxylates; castor oil alkoxylates; the partial esters derived from long chain fatty acids and hexitol anhydrides and their alkoxylates; long chain alcohol polyglycol ether acetals; alcohol sugar acetals; and the lecithins. Detergents such as "Tween"20, "Nonidet" P40 and "Triton" X100 (Trade Marks) have been found particularly useful.

Industrial Applicability

It will be evident to those skilled in the art that the products and methods of the invention find particular use in the medical and veterinary fields for the analysis of PAF.

Preferred Embodiments

Embodiments of the present invention will now be described by way of example only.

Example 1

Preparation of 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl-3-phosphorylcholine

1. 1,1-Dimethoxycyclohexane.

A mixture of cyclohexanone (52 ml, 0.5 mol), trimethylorthoformate (66 ml, 0.6 mol), methanol (51 ml, 1.26 mol) and concentrated H_2SO_4 (1 drop) was refluxed for 18 hours. A solution of sodium methoxide in methanol was added until the mixture was neutral, and the mixture was fractionally distilled. 1,1-Dimethoxycyclohexane was obtained from the fraction b.p. 162-164°C (50.6 g, 70%).

2. 1-Methoxycyclohexene.

1,1-Dimethoxycyclohexane (25 g, 0.174 mol) was heated with p-toluenesulfonic acid (35 mg) at 140°C for 3 hrs. Methanol was distilled off during the reaction. The residue was fractionally distilled, yielding 1-methoxycyclohexene (15.2 g, 80%) b.p. 144-146°C.

3. Methyl 6,6-dimethoxyhexanoate.

A solution of 1-methoxycyclohexene (4.5 g, 0.04 mol) in methanol (140 ml) was ozonolysed at 0°C until the uptake of ozone ceased. The solution was degassed and a suspension of reduced $Pd/CaCO_3$ (1.0 g) catalyst in methanol (30 ml) was added. The mixture was filtered through celite, and the filtrate was evaporated. Trimethylorthoformate (7 ml, 0.06 mol), methanol (5 ml, 0.12 mol) and conc.H_2SO_4 (1 drop) were added to the residue. After 17 hours, the mixture was neutralized with sodium methoxide solution and then fractionally distilled. Methyl 6,6-dimethoxyhexanoate was collected as the fraction b.p. 80-90°C/1.0 mm (4.1 g, 54%).

4. 6,6-Dimethoxyhexan-1-ol

To a stirred mixture of lithium aluminium hydride (3.8 g, 0.1 mol) in ether (80 ml) under nitrogen, was added methyl 6,6-dimethoxyhexanoate (15.0 g, 0.079 mol) in ether (50 ml) at a rate to maintain reflux (ca.1.5 hr). The mixture was further refluxed for 1.5 hrs., and then cooled to 0°C. Sodium hydroxide solution (13 ml, 7 M) was added dropwise while cooling in ice. After stirring for 1 hour, the mixture was filtered through a layer of magnesium sulfate. The residue was washed with ether, and the combined filtrates were evaporated. The residue was

subjected to "suction" chromatography. 6,6-Dimethoxyhexan-1-ol was eluted with 25% ethyl acetate in light petroleum (10.3 g, 80%).

5. 2-O-Acetyl-3-O-benzyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol

Sodium hydride dispersion (0.377 g, 12.6 mmol, 80% in oil) was washed with dry ether under nitrogen. The residue was resuspended in dry DMF (30 ml), and 6,6-dimethoxyhexan-1-ol (1.62 g, 10 mmol) was added. The mixture was heated at 80°C for 1.25 hr., during which time the sodium hydride reacted. (R)-1-(Benzyloxy)-2,3-epoxypropane (1.64 g, 10 mmol) was added and heating was continued for 2 hr. Upon cooling, water (100 ml) was added and the mixture was extracted with ether (100 ml, 2 x 40 ml). The combined extracts were washed with water (2 x 80 ml) and brine (100 ml), dried ($MgSO_4$) and evaporated. The residual oil (2.8 g) was dissolved in chloroform (36 ml), and cooled to 0°C. Pyridine (3.5 ml, 43 mmol) and freshly distilled acetyl chloride (0.94 ml, 13.2 mol) were added. The mixture was stirred for 0.5 hr. at 25°C, then 2 hr. at room temperature

(RT). Ice water (100 ml) was added and the layers separated. The aqueous layer was extracted with chloroform (2 x 40 ml), and the combined organic phases were washed with water (100 ml) and brine (100 ml), dried ($MgSO_4$) and evaporated. The residue was subjected to chromatography and the product was eluted with petroleum ether-ethyl acetate (9:1). Evaporation of this fraction yielded the product as a colorless oil (1.82 g, 50%) b.p. 170°C/0.2 mmHg ($C_{20}H_{32}O_6$ requires C, 65.19; H, 8.75%, Found: C, 65.06%; H,8.66%), $[\alpha]_D$ + 1.98° (c 5.06, benzene). ^{1}H N.M.R.δ : 7.36, m,5, ArH; 5.17,q.1,J 5.0 Hz, H2; 4.50,d,2,J 2.5 Hz, benzyl; 4.36, t,1,J 5.7 Hz, -CH(OMe_2); 3.62,d,2,J 5.0 Hz, H3; 3.58,d,2,J 5.2 Hz, OCH_2-; 3.48-3.39,m,2,H1; 3.31,s,6,OCH3; 2.12,s,3,$COCH_3$; 1.72-1.26,m,8,-CH_2-. Mass spectrum: m/e 337, 305, 287, 245, 229, 215, 207, 146, 117, 113, 111, 91, 81, 75, 72.

6. 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol

2-O-Acetyl-3-O-benzyl-1-O-(6',6'-dimethoxyhexyl)-sn--glycerol (369 mg, 1.0 mmol) was hydrogenated in THF (10 ml) over Palladium/carbon (14 mg, 10%) until the uptake of hydrogen ceased (approx. 2.5 hr.). The solution was filtered through celite, and the filtrate was evaporated to yield a colourless oil (278 mg, 100%) which was used immediately. ^{1}H N.M.R. δ : 5.04,q,1,J 5.0 Hz,H2; 4.40,t,1,J 5.7 Hz, -CH($OMe)_2$; 3.84,d,2,J 5.0 Hz, H3; 3.65,d,2,J 5.2 Hz, OCH_2-; 3.56-3.44,m,2, H1; 3.35,s,6, OCH_3;

2.5,s(b),1,OH; 2.14,s,3, $COCH_3$; 1.7-1.3,m,8, $-CH_2-$.

7. 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl 3-phosphorylcholine

To a stirred, cold (0°C) solution of distilled triethylamine (0.35 ml, 2.5 mmol) in dichloromethane(4 ml) under nitrogen, was added distilled phosphorous oxychloride (0.11 ml, 1.2 mmol) and then 2-O-acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol (278 mg, 1.0 mmol) in dichloromethane (5 ml). The solution was stirred for 1 hr. at RT, and choline tosylate (465 mg, 1.7 mmol) in pyridine (10 ml) was added. Stirring was continued for 17 hrs. at RT. Sodium bicarbonate (0.4 g) and water (1 ml) were added and the mixture was evaporated at 30°C. The residue was extracted several times with chloroform (total 40 ml) and filtered. The filtrate was evaporated to yield a semi-solid residue (1.3 g).

An anion exchange column was prepared from DE-32 celluose (5.5 g) in acetic acid, and washed successively with methanol, methanol/chloroform (1:1) and chloroform. The mixture (1.3 g) was applied to the column in a small volume of chloroform, and was then eluted with chloroform (100 ml), then methanol in chloroform (100 ml each of 1.5%, 3%, 4.5%, 6% v/v). The product was contained in the fractions 3-6% methanol in chloroform, as determined by t.l.c ($CHCl_3/MeOH/H_2O$

60:35:5). Evaporation of these combined fractions yielded a pale yellow semi-crystalline material (0.21 g), which was contaminated with a tosylate salt (approx. 30%). ^{1}H N.M.R. δ : 5.13,m,1,H2; 4.37,t,1,J 5.7 Hz,-$CH(OMe)_2$; 4.3-3.2,m,all other protons on Cα to O or N; 2.06, s,3, $COCH_3$; 1.7-1.3,m,8,-CH_2-. ^{13}C N.M.R. δ 170.49,s, C=O; 104.25, s, -$CH(OMe)_2$; 71.90,d,J 8.0 Hz, C2; 71.17,s,-CH_2O(or N); 69.00,s,-CH_2O(or N); 65.76,s,-CH_2O (or N); 63.76,d, J 5.1 Hz; -CH_2OP; 59.03,d,J 4.4 Hz, -CH_2OP; 53.78,s,-$N^+(CH_3)_3$; 53.38,s,OCH_3; 32.24,s,-CH_2-;21.00,s, $COCH_3$.

Example 2

Preparation of 2-O-Acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine

Crude 2-O-acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl-3-phosphorylcholine (130 mg) was suspended in ethyl acetate (9 ml) and aqueous trifluoroacetic acid (TFA) (170 μl, 90%) was added. The mixture was allowed to stand at RT for 1.5 hr. and 4°C for 17 hrs., until the deprotection was complete by t.l.c. Toluene (9 ml) was added and the mixture evaporated. The residue was repeatedly evaporated from ethyl acetate/toluene (1:1) (10 ml) and alternatively from toluene (10 ml). The mixture was

chromatographed on silica gel (70-230 mesh) and the product was eluted with $CHCl_3/MeOH/H_2O$ (40:60:10). Evaporation of the appropriate fractions yielded a colorless oil (50 mg). ^1H N.M.R. δ : 9.78,t,1,J 2.0 Hz, CH=O; 5.1,m,1,H2; 4.4-3.2,m, all other protons on C α to O or N; 2.46, dt,2,J 2.0 & 7.0 Hz, $-CH_2-CHO$; 2.08,s,3,$COCH_3$; 1.7-1.3,m,6,$-CH_2-$. ^{13}C N.M.R. δ: 176.05,s, -CHO; 170.79,s, $-OCOCH_3$; 72.07,s,C2; 71.21,s,$-CH_2O$ (or N); 69.27,s, $-CH_2O$ (or N); 66.12,s, $-CH_2O$ (or N); 64.11,s, $-CH_2OP$; 59.38,s, $-CH_2OP$; 54.20,s,$-N^+(CH_3)_3$; 43.78,s,$-CH_2CHO$; 29.28,s, $-CH_2-$;25.64,s,$-CH_2-$; 21.80,s,$-CH_2-$; 21.26,s,$COCH_3$.

Example 3

Coupling 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine to methylated BSA(PAF-BSA)

Methylated bovine serum albumin (250 mg) was dissolved in methanol (90 ml) and 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine (25 mg) in methanol (5 ml) was added. The solution was left at RT for 0.5 hr., and then sodium cyanoborohydride (100 mg) was added. The pH of the solution was adjusted to 5 with 1M HCl. After standing for 16 hr. at RT, the mixture was evaporated. The residue was dispersed in water (90 ml) and dialysed against distilled water (20 l). The dialysate was

freeze-dried to yield a fluffy white material (238 mg). This material was assayed for phosphorous content, which was found to be 100 nanomoles per mg.

Example 4

Coupling 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl -3-phosphoryl-choline to polylysine (PAF-PL)

2-O-Acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphoryl-choline was coupled to the polyvalent synthetic polypeptide polylysine following essentially the same procedure as that described in Example 3.

Example 5

Inactivation of PAF-Acetylhydrolase

The following experiments demonstrate that PAF-acetyl-hydrolase can be deactivated by the addition of detergents.

Materials

PAF (from bovine heart lecithin) and "Tween" 20 (polyoxy-ethylene sorbitan monolaurate) were from Sigma (St. Louis, Mo., USA). Human sera albumin (HSA) was from Commonwealth Serum Laboratories (Melbourne, Australia).

Serum

Blood was collected from normal human donors by venipuncture, allowed to clot and the serum collected. Serum was stored at -20°C until used. Similarily, rabbit serum was obtained from the ear veins of normal rabbits.

Platelet-Rich Plasma

Whole blood was collected from normal human donors, who had taken no medication for at least 10 days before venipuncture, and mixed with 0.1M trisodium citrate (0.1 vol). Platelet-rich plasma was produced by centrifugation (10 min, 600 r.p.m.) and was used within 1 hour.

Dilution of Sera

Sera were diluted 1 in 100 in either PBS or 0.1% "Tween" in PBS (v/v). Diluted acid-treated sera were prepared by mixing sera (1 vol.) with 0.1M citrate buffer pH 3.0 (2 vol.), and then 15 minutes later with PBS (98 vol.).

Determination of PAF-acetylhydrolase activity

Diluted serum (50 μl) was incubated with 3.7×10^{-6} M PAF (in 2.5% HSA) (50 μl) for 27 hours at 25°C. The solution (50 μl) was then tested for platelet aggregation activity at 37°C in a Payton dual

channel aggregometer using human platelet-rich plasma (500μl).

RESULTS AND DISCUSSION

Two human sera and two rabbit sera, each with added PAF, were diluted by the three methods (PBS, "Tween" and acid-treated) and were then tested for acetylhydrolase activity. The results were in the form of light-transmission tracings from the aggregometer. After 27 hours incubation, PAF was destroyed in all sera diluted with PBS whereas the sera diluted in 0.1% "Tween" showed no inactivation of PAF. The "Tween"-diluted sera were tested for platelet aggregating activity, but no aggregation was observed. As a control for the above experiment, PAF was incubated with PBS or 0.1% Tween in PBS. In these experiments platelet aggregation activity was retained.

Disparity between human and rabbit sera was found when the sera were treated with acid. Whereas, rabbit sera no longer destroyed PAF, acid-treated human serum still had acetylhydrolase activity. Human and rabbit sera appear to have the same buffering capacity, so the disparity probably arises from varying acid-sensitivities of the two acetylhydrolases.

These results show that "Tween" 20 inactivates PAF-acetylhydrolase. Dilution in "Tween" is thus a simple and

mild method of inactivating PAF-acetylhydrolase and this finding will be of great importance in immunoassay procedures used to measure PAF in biological fluids.

Example 6

Preparation of PAF-antibodies

2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphoryl-choline coupled to methylated bovine serum albumin prepared as described in Example 3 (PAF-BSA) was used as an antigen in rabbits and the immunoglobulin fraction was isolated from the rabbit anti-PAF serum produced by affinity chromatography on "Sepharose"/protein A.

The presence of PAF-antibodies in the isolated immunoglobin fraction was determined by a direct binding assay showing binding to tritium labelled PAF (^{3}H-PAF) as described below.

A sample of the immunoglobulin fraction (Ig) was mixed in an assay tube with a mixture (3-5mg) of "Sepharose" (solid support) and protein A (a ligand to link the antibody to solid support) and ^{3}H-PAF in a total volume of 50 to 100 μl and incubated at room temperature overnight. The resulting mixture was centrifuged, washed twice with phosphate buffered saline containing 0.1% "Tween" 20, centrifuged and the sediment transferred in

water (200 µl) to the liquid scintillant "Aquasol" (3ml) and counted in a liquid scintillation counter.

The results, tabulated below, indicate significant uptake of ^{3}H-PAF by the immunoglobulin isolated from rabbits treated with the PAF-BSA antigen in comparison to "normal" immunoglobulin isolated from control rabbits.

Rabbit No	Ig (µg)	^{3}H-PAF (cpm)	Assay Count (cpm)	^{3}H-PAF Uptake (%)
1	20	28,123	5,046	17.9
1	10	28,123	5,124	18.2
1	5	28,123	3,967	14.1
2	20	28,123	4,449	15.8
2	10	28,123	3,001	10.7
2	5	28,123	2,189	7.8
Control	20	28,123	326	1.2
Control	10	28,123	492	1.7
Control	5	28,123	281	1.0
None	0	28,123	140	0.5

*"Sepharose", "Tween" and "Aquasol" are Trade Marks.

Example 7

The following experiments demonstrate the use of PAF-antibodies of the present invention in a competition or inhibition assay with a known quantity of labelled PAF and known quantities of unlabelled PAF or PAF analogues of the invention which can be used to establish standard plots from which the quantity of PAF in sample can be determined. They also demonstrate the binding of the PAF-antibodies of the invention to PAF and the PAF analogues of the invention (e.g. PAF-PL of Example 4) in comparison to lyso-PAF, lecithin and lyso-lecithin.

A standard quantity of immunoglobulin containing PAF-antibodies (Ig) prepared as described in Example 6 was mixed in an assay tube with a mixture (3-5mg) of "Sepharose" and protein A, ^{3}H-PAF (22,676 cpm), and a sample of a "test" substance for competitive binding to PAF-antibodies in a total volume of 100 to 200 µl and the mixture was incubated at room temperature overnight. The resulting mixture was centrifuged, washed twice with phosphate buffered saline containing 0.1% "Tween" 20, centrifuged and the sediment transferred in water (200µl) to the liquid scintillant "Aquasol" (3ml) and counted in a liquid scintillation counter.

The results, tabulated below, indicate:

(i) PAF-antibodies of the present invention may be used in a competition assay with known amounts of radiolabelled PAF and PAF to develop a standard plot for the quantitative determination of PAF by competition assay; and

(ii) the specific binding of the PAF-antibodies of the invention to PAF and the PAF-analogues of the invention (e.g. PAF-PL)

TEST SUBSTANCE		ASSAY COUNT	ASSAY/CONTROL
Name	ng	cpm	%*
PAF	5,000	228	4.1
PAF	500	598	18.6
PAF	50	1,602	57.8
PAF	5	2,316	85.7
PAF	0.5	2,561	95.2
PAF-PL	27,000	435	12.2
PAF-PL	2,700	662	21.1
PAF-PL	270	429	11.9
PAF-PL	27	1,329	47.1
PAF-PL	2.7	2,338	86.5

lyso-PAF	5,800	2,746	-
lyso-PAF	580	2,744	-
lecithin	5,000	2,994	-
lecithin	500	2,545	99.6
lyso-lecithin	5,000	2,658	99.0
lyso-lecithin	500	2,668	99.4
Control	0	2,683	100.0
No Ig	0	123	-

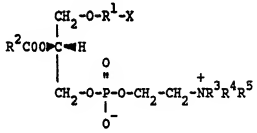
---

* $\frac{\text{Assay Count - Assay Count No Ig}}{\text{Assay Count Control - Assay Count No Ig}} \times 100$

i.e. $\frac{(\text{Assay Count} - 123) \times 100}{2560}$

CLAIMS:

1. Compounds of general formula (I):



wherein:

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;

X is hydrogen; or

(2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynlene linking group optionally substituted by tritium or radioactive iodine;

X is selected from:

(a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; and

(b) the group -A-B wherein A is a linking group selected from the groups $-NR^6-$, -COO-, -OCO-, $-CONR^6-$, $-NR^6CO-$, -NH-CS-NH- and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and B is selected from:

(i) monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of

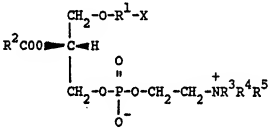
molecular weight of at least 2000; and

(ii) a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl;

and mixtures of the compound of formula I and its enantiomer.

2. Antigenic PAF analogues of general formula (I)



wherein:

R^1 is a C_2 to C_{25} alkylene or alkynylene linking group;

X is the group -A-B wherein:

A is a linking group selected from $-NR^6-$, -COO-, -OCO-, $-CONR^6$, $-NR^6CO-$ and -S-S wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000 which are capable of eliciting an antigenic response; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

3. Antigenic PAF analogues according to claim 2 wherein:

R^1 is selected from straight chain C_4 to C_{16} alkylene;

X is a group -A-B wherein:

A is selected from $-NR^6-$, -COO-, -OCO-, $-CONR^6-$ and $-NR^6CO-$ wherein R^6 is hydrogen or methyl; and

B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight at least 5000 which are capable of eliciting an antigenic response; and

R^2 to R^5 are independently selected from C_1 to C_3 alkyl.

4. Antigenic PAF analogues according to claim 2 or claim 3 wherein:

R^1 selected from straight chain C_4 to C_8 alkylene;

X is a group -A-B wherein:

A is selected from -NH- and -COO- ; and

B is selected from monofunctional and polyfunctional protein and peptide groups of molecular weight at least 10,000 which are capable of eliciting an antigenic response; and

R^2 to R^5 are each methyl.

5. Antigenic PAF analogues according to any one of claims 2 to 4 inclusive wherein:

R^1 is hexylene;

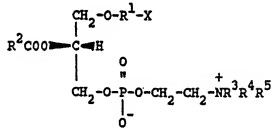
X is a group -A-B wherein:

A is -NH-; and

B is selected from a protein residue derived from bovine serum albumen and a peptide residue derived from polylysine; and

R^2 to R^5 are methyl.

6. Labelled PAF analogues of general formula (I)



(I)

wherein

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine; X is hydrogen; or

(2) R^1 is a C_2 to C_{25} alkylene, alkenylene, or alkynylene linking group;

X is a group of formula -A-B wherein:

A is a linking group selected from $-NR^6-$, -COO-, -OCO-, -OCO-, $-CONR^6$, $-NR^6CO-$, -NH-CS-NH- and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl;

B is a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

7. Labelled PAF analogues according to claim 6 wherein:

R^1 is selected from straight chain C_4 to C_{16} alkylene;

X is a group of formula -A-B wherein:

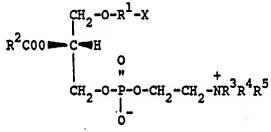
A is selected from $-NR^6-$, -COO-, -OCO-, $-CONR^6-$ and $-NR^6CO-$ wherein R^6 is hydrogen or methyl; and

B is labelled group selected from:

radiolabelled groups based on ^{125}I-histamine, ^{125}I-tyramine, ^{125}I-tyrosine methyl ester and ^{125}I-Bolton Hunter Reagent; enzymic labels; and photometric labels; and

R^2 to R^5 are independently selected from C_1 to C_3 alkyl.

8. Compounds of general formula (I) which are intermediates for the preparation of PAF analogues



(I)

wherein:

R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group; and

X is selected from the group consisting of formyl, carboxy, di(C_1 to C_6 alkoxy)methyl, N-C_1 to C_6 alkyl)amino, hydroxy and mercapto.

9. Compounds according to claim 8 wherein:

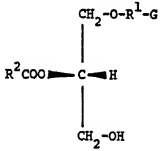
R^1 is selected from straight chain C_4 to C_{16}; and

X is selected from formyl, carboxy, dimethoxymethyl and hydroxy.

10. A process for the preparation of compounds of general formula (I) which process comprises:

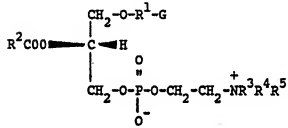
(a) reacting:

a compound of general formula (II)



II

wherein R^1 and R^2 herein before defined and G is selected from di(C_1 to C_6 alkoxy)-methyl and groups which may be reacted, to give a group selected from formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, amino, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; a phosphorylation agent; and an N,N,N-tri(C_1 to C_6 alkyl) ethanolamine derivative to give a compound of general formula (III)

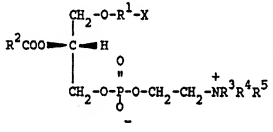


(III)

(b) reacting the product of (a) to convert group G as hereinbefore defined to a group selected from formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, amino, N-C_1 to C_6 alkylamine, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto and to introduce the desired group X.

11. Supported PAF analogues comprising:

PAF analogues of general formula (I)



I

wherein:

(1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;

X is hydrogen; or,

(2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;

X is selected from:

(a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy isothiocyanato, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; and

(b) the group - A-B wherein A is a linking group selected from the groups $-NR^6-$, -COO-, -OCO-, $=CONR^6$, $-NR^6CO-$, -NH-CS-NH- and -S-S- wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl; and a solid support material upon which said PAF analogues are covalently bound.

12. PAF antibodies prepared using as antigen:

(a) PAF adsorbed onto or non-covalently bound to a monofunctional or polyfunctional protein, peptide, carbohydrate, lipid or a derivative thereof of molecular weight at least 2000 and capable of eliciting an antigenic response; or

(b) the antigenic PAF analogues of general formula (I) as defined according to any one of claims 2 to 5 inclusive.

13. PAF or antibodies prepared using as antigen an antigenic PAF analogue of general formula (I) as defined according to claim 4 or claim 5.

14. A method for the preparation of PAF antibodies which method comprises:

introducing an antigen selected from:

(a) PAF adsorbed onto or non-covalently bound to a monofunctional or polyfunctional protein,

peptide, carbohydrate, lipid or a derivative thereof of molecular weight at least 2000 and capable of eliciting an antigenic response; and

(b) the antigenic PAF analogues of general formula (I) as defined according to any one of claims 2 to 5 inclusive;

into an animal; and

isolating the antibodies produced in response to said antigen.

15. A method for the preparation of PAF antibodies which method comprises:

introducing an antigen selected from the antigenic PAF analogues of general formula (I) as defined according to claim 4 or claim 5 into an animal; and isolating the antibodies produced in response to said antigen.

16. PAF antibodies as defined according to claim 12 or claim 13 which have been labelled with a radioactive, enzymic or photometric label.

17. PAF antibodies as defined according to claim 12 or claim 13 which are polyclonal.

18. PAF antibodies as defined according to claim 12 or claim 13 which are monoclonal.

19. A method for the immunoassay of PAF in biological fluids which method comprises using a PAF antibody as defined according to any one of claims 12, 13 and 16 to 18 inclusive.

20. A method for the immunoassay of PAF in biological fluid wherein said biological fluid is diluted with a detergent before subjecting said biological fluid to immunoassay.

21. A method according to claim 20 wherein said detergent is a non-ionic detergent.

22. A method according to claim 20 or 21 wherein said detergent is selected from the group consisting of: polyalkylene glycols; alcohol, phenol and alkylphenol alkoxylates; castor oil alkoxylates; the partial esters derived from long chain fatty acids and hexitol anhydrides and their alkoxylates; long chain alcohol polyglycol ether acetals; alcohol sugar acetals; and the lecithins.

23. A kit for the immunoassay of PAF in biological fluid said kit comprising PAF antibodies as defined according to any one of claims 12, 13 and 16 to 18 inclusive.

24. Compounds of general formula (I) according to any one of claims 1 to 9 inclusive substantially as herein described with reference to Examples 1 to 4.

25. Method according to claim 10 for the preparation of compounds of general formula I substantially as herein described with reference to Examples 1 to 4.

26. PAF antibodies according to any one of claims 12, 13 and 16 to 18 inclusive substantially as herein described with reference to Example 6.

27. Method according to claim 14 or claim 15 for the preparation of PAF-antibodies substantially as herein described with reference to Example 6.

28. Method for the immunoassay of PAF in biological fluids according to any one of claims 19 to 22 inclusive substantially as herein described with reference to Example 6 or Example 7.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00084

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07F 9/10, G01N 33/92, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched [5]	
Classification System	Classification Symbols
IPC	C07F 9/10, G01N 33/92, G01N 33/16, C07K 15/12, C07G 7/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [6]

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [8]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A, 4370311 (ILEKIS) 25 January 1983 (25.01.83) See column 2 lines 11-18	(20-22)
A	Journal of Immunology Vol. 134 No.2 (1985) M. Odo et al, "Molecular Species of Platelet-Activating Factor Generated by Human Neutrophils challenged with Ionophore A23187" pages 1090-3	
A	US,A, 3708558 (KNY) 2 January 1973 (02.01.73)	
A	CA,A, 1169433 (GOVERNMENT OF THE UNITED STATES OF AMERICA) 19 June 1984 (19.06.84)	
A	Patent Abstracts of Japan, C-9, page 117, JP 55-28955 (TOYAMA KOGAKU KOGYO K.K.) 29 February 1980 (29.02.80)	
A	US,A, 4329302 (HANAHAN) 11 May 1982 (11.05.82)	
A	Chemical Abstracts, Volume 104, No.5 issued 1986, Lakin K. et al, "Activation of Rabbit Platelets induced by 1-O-alkyl-2-O-acetyl-sn-glycerophosphocholine" see page 388, abstract No. 32325s, Byull. Eksp. Biol. Med. 1985 100(10)410-12 (Russ).	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 June 1987 (18.06.87)	(22.07.87) 2 JULY 1987
International Searching Authority: Australian Patent Office	Signature of Authorized Officer: J.G. HANSON

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

(1) Claims 1 to 11, 12(b),13,14(b),15 to 19,and 23 to 28

(2) Claims 12(a) and 14(a)

(3) Claims 20 to 22

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00084

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
US 3708558	BE	763578	CH	542247	DE	2009341
	ES	388446	GB	1280788	IL	36299
	NL	7102495	YU	343/71	ZA	7101241
US 4329302	US	4504474	US	4551446		

END OF ANNEX

Search Notes

Application/Control No.	Applicant(s)/Patent under Reexamination	
10/814,194	FROSTEGARD, JOHAN	
Examiner	**Art Unit**	
Lisa V. Cook	1641	

SEARCHED			
Class	Subclass	Date	Examiner
435	7.1, 7.21, 7.92, 7.9	12/7/05	LVCook
436	501, 517	12/7/05	LVCook
514	75	12/7/05	LVCook
updated	oo abort	6/6/06	LVCook

INTERFERENCE SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES (INCLUDING SEARCH STRATEGY)		
	DATE	EXMR
STN – biosis, caplus, uspatfull, medline, compendex, jpplo East and west Patent database Palm inventor name	12/7/05	LVCook
Search and related applications 09/720,967 – us Patent # 6,780,605 application # 10/814,195	12/7/05	LVC
updated oo abort	6/6/06	LVC

Index of Claims

Application/Control No.	Applicant(s)/Patent under Reexamination
10/814,194	FROSTEGARD, JOHAN
Examiner	**Art Unit**
Lisa V. Cook	1641

√	Rejected	–	(Through numeral) Cancelled	N	Non-Elected	A	Appeal
=	Allowed	+	Restricted	I	Interference	O	Objected

Claim		Date								
Final	Original	12/7/05 LVCook	6/6/06 LVCook							
	1	✓	✓							
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THE DEGRADATION OF PLATELET-ACTIVATING FACTOR IN SERUM AND ITS DISCRIMINATIVE VALUE IN ATHEROSCLEROTIC PATIENTS

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ABSTRACT

Platelet-activating factor (PAF) is transformed in vivo rapidly into the biologically inactive lyso-PAF. This reaction as well as lipid parameters were quantified in serum from 40 survivors of myocardial infarction and 36 healthy controls matched for age and body weight. The PAF-degrading capacity was 23% ($p<0.001$) higher in patients compared with the control group. Using the degradation of PAF as an univariate discriminator more than 70% of subjects were classified correctly. This is comparable with the discriminatory value of the best lipid variables, apolipoprotein B and HDL-cholesterol. Statistically significant differences in the degradation of PAF were found also by comparing subgroups which were matched for plasma levels of total cholesterol, VLDL/LDL-cholesterol or apolipoprotein B. The ratio HDL-cholesterol/degradation of PAF which is increased by 48 % ($p<0.0001$) in the case group was identified as an additional good discriminator between both groups. In contrast, platelet aggregation tests which were performed in acetylsalicyclic acid treated platelet-rich plasma discriminated poorly between patients and controls.

Key words: Platelet-Activating Factor, Lipoproteins, Platelet Function Tests, Atherosclerosis

INTRODUCTION

Platelet-activating factor (PAF, 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine) is an extremely potent lipid mediator [1] which is considered to be involved in various inflammatory, respiratory, and cardiovascular disorders [2].

The effects of PAF are limited *in vivo* by a rapid removal of the 2-O-acetyl group [3]. This reaction is catalyzed by a PAF-specific acetylhydrolase [4] which is found in various tissues and cells as well as in serum or plasma. The plasmic PAF-acetylhydrolase which has been purified recently [5] is strongly bound to lipoproteins [6-8] and has properties somewhat different from those of the cellular enzyme [9]. Investigations on the uptake and degradation of PAF by individual lipoproteins revealed that the lipoproteins play a more complex role in the degradation of PAF than simply binding PAF-acetylhydrolase [10]. Moreover, we recently have shown, that the degradation of PAF in serum and plasma correlates highly significant with the lipoprotein profile [7] and is increased in patients suffering from peripheral vascular disease [11]. Abnormal high PAF-acetylhydrolase activities were found also in plasma of patients with familial LCAT deficiency [12] and a case of Tangier disease [13] which is characterized by the virtual absence of high density lipoproteins.

In view of these manifold relationships between the lipoprotein profile and the degradation of PAF and the well known role of lipoproteins as important risk factor for atherosclerosis [14], the present study was undertaken to establish whether serum PAF-acetylhydrolase is useful to discriminate between patients suffering from cardiovascular diseases and healthy subjects.

For this purpose the degradation of PAF and various lipid variables were measured in serum from survivors of myocardial infarction and an age- and body weight matched control group. Additionally, some functional parameters of platelets representing one of the target cells of PAF which play also an important role in atherogenesis [15], were included in this study.

MATERIALS AND METHODS

Chemicals and reagents: ^{14}C-PAF was prepared by reacting 1-O-hexadecyl-rac-glycero-3-phosphocholine with ^{14}C-acetic anhydride (30.5 MBq/mg, Isocommerz GmbH, Berlin, GDR) in anhydrous pyridine as described previously [16]. The labelled compound was chromatographed on silica gel resulting in a radiopurity of greater than 95%. It was dissolved in albumin-PBS (2.5 mg human serum albumin per ml of phosphate-buffered saline, pH 7.4), stored at -20°C, and further diluted with albumin-PBS immediately before use.

Subjects: Two groups of male subjects, 40 patients with clinical evidence of atherosclerotic diseases and 36 age- and body weight-matched healthy subjects were included into this study. The case group was recruited from outpatients of the

Clinic of Internal Medicine of the Medical Academy of Erfurt and had survived a myocardial infarction at least one year before their entry into this study. Myocardial infarction was documented by specific criteria including electro cardiographic changes, elevated serum enzymes, and typical symptoms. The control group consisted of healthy volunteers who had no known history of symptoms of heart disease [17].

Blood sampling und platelet preparation: Blood was always taken by venipuncture after an overnight fasting. Serum and heparin plasma were separated by centrifugation and stored frozen until further analysis. To prepare platelets, blood was collected into 0.1 volume citrate/ASA (0.11 mol/l trisodiumcitrate / 0.5 mmol/l acetylsalicylic acid) and centrifuged for 10 min at 200xg. The platelet-rich plasma (PRP) was aspirated, adjusted to 2.5×10^8 platelets/ml with autologous platelet-poor plasma (PPP), and stored in tightly stoppered plastic tubes at room temperature. PPP was obtained by centrifugation of PRP for 10 min at 1400xg. Aggregation tests were started 90 min after blood collection.

PAF-degrading capacity: The degradation of PAF in serum was measured under standard conditions by a method similar to that described by BLANK et al. [4]. 50 µl serum dilution (1:19) were added to 0.5 ml of 11 µM ^{14}C-PAF at a temperature of 37°C. After 5 and 10 min the reaction was terminated by transferring aliquots of 0.2 ml into 0.6 ml of ice-cold chloroform/methanol (2/1,v/v). The samples were mixed and then centrifuged at 8000xg for 3 min. The upper phase was removed, washed with chloroform and finally the amount of ^{14}C-acetate was assayed by liquid szintillation counting. Mean values of four separate incubations were used.

Lipids and apolipoproteins: Triglycerides and cholesterol were measured in heparin plasma by enzymatic methods using commercially available test kits (Boehringer, Mannheim, FRG). Cholesterol of the high density lipoproteins (HDL) was measured after precipitation of very low density lipoproteins (VLDL) and low density lipoproteins (LDL) with phosphotungstate-MgCl$_2$. VLDL/LDL-cholesterol and LDL-cholesterol were then calculated by difference and according to the formula of FRIEDEWALD [18], respectively. The apolipoproteins (apo) A-I and B were measured by immunonephelometric methods as described elsewhere [19,20].

Platelet function tests: Platelet aggregation was measured turbidimetrically according to BORN [21] using a two-channel aggregometer, model KLVI 840 (Elvi Logos, Milan, Italy). After calibration with PRP and PPP the aggregation was triggered by adding 20 µl solutions of PAF, ADP or collagen to 200 µl PRP at a cuvette temperature of 37°C and a constant stirring speed of 900 rpm. Platelet responses were measured as maximum increase in light transmission (AT) occurring within 1 and 3 min after adding the inducer. Additionally, ADP- and PAF-concentrations response curves were constructed and the concentration required to produce a half-maximum aggregation response (EC_{50}) was red by interpolation. ADP, PAF, and collagen were used in final concentrations of 0.5-15 µM, 0.02-10 µM, and 5 µg/ml, respectively.

Moreover, platelet responses were studied in whole blood . For this purpose anticoagulated blood was placed into an aggregometer cuvette and stirred for 10 min. Aliquots were taken before and after stirring, transferred into ammonium oxalate/xylocitin and counted for the number of single platelets by use of phase contrast microscopy. Spontaneous aggregation was expressed as the percentage loss of single platelets obtained after stirring.

Statistical analyses: The variables of both groups were tested for normal distribution using the KOLMOGOROV-SMIRNOV test. Statistical significances between group means were assessed by the two-tailed STUDENT's t test and in one case by the paired WILCOXON's rank test. Linear correlations were calculated to evaluate relationships between various parameters. Univariate discriminant analysis was performed by setting cut off points according to the criterion of minimal apparent error rate representing the sum of falsely positive and falsely negative classified individuals.

RESULTS

Seventy six male subjects, 36 healthy volunteers and 40 atherosclerotic patients were included in this study. The age of the controls was 53±5 years and that of the patients was 52±7 years. Broca index was 107±12% in both groups. The subjects of the control group did not take any drug for at least two weeks prior to blood sampling. Coronary and peripheral arterial diseases were excluded by physical examination as well as by electro cardiographic examination during and after exercise. The patients had survived a myocardial infarction 1-18 years before their entry into this study and were administered with aspirin (7 cases), calcium channel blockers (17 cases) and nitrovasodilators (18 cases). They did not suffer from other diseases particularly essential hypertension and diabetes mellitus and were refrained from taking ß-blockers 2 weeks prior to blood sampling.

The first step of our analysis was to characterize the distribution and location of the biochemical parameters in the two groups. All variables were distributed normally and fulfilled the necessary conditions for the application of parametric statistical procedures. There was also a considerable overlapping of most variables for patients and controls. In spite of this fact, a comparison of the mean values revealed a series of significant differences between both groups (see Table 1). Thus, the degradation of PAF was 23% higher in serum from the case group compared with the controls. Also the serum lipoprotein profile of the patients was characterized by the typical abnormalities. Concentrations of triglycerides, VLDL/LDL-cholesterol and apo B were significantly increased and those of HDL-cholesterol and apo A-I significantly lowered. The differences observed between both groups in total- and LDL-cholesterol were statistically not significant.

To evaluate the discriminatory power of the various parameters, cut-off points and apparent error rates were calculated

(see also Table 1). According to the criterion of minimal error rate, apo B, HDL-cholesterol, and the degradation of PAF were identified as the best discriminators between patients and controls. Using these variables as single parameters more than 70% of the subjects were classified correctly. Triglycerides, VLDL/LDL-cholesterol, and apo A-I which were also significantly different in both groups resulted in higher error rates compared to the former variables.

TABLE 1

PAF-Degrading Capacity (nmol/ml x min), Lipids (mmol/l) and Apolipoproteins (g/l) of Normal Subjects and Survivors of Myocardial Infarction

Parameter	Controls (N = 36)	Patients (N = 40)	COP	AER (%)	p
Degradation of PAF	31.8±8.26	39.0±7.38	33.4	29	***
Total cholesterol	6.49±1.30	6.74±0.81	6.53	34	n.s.
HDL-cholesterol	1.45±0.27	1.19±0.26	1.28	29	***
VLDL/LDL-cholesterol	5.04±1.39	5.55±0.77	5.13	32	*
LDL-cholesterol	4.34±1.27	4.55±0.78	4.36	40	n.s.
Triglycerides	1.55±0.69	2.21±1.09	1.60	34	**
Apo A-I	117±18	104±14	108	36	***
Apo B	96±29	112±15	103	25	**

Means ± S.D. are shown. N = number of subjects; COP = cut-off point; AER = apparent error rate; n.s. = not significant ($p>0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$;

The mean PAF-degrading capacity of serum from myocardial infarction survivors was found to be significantly increased also in a comparison with that of controls who had identical serum levels of total cholesterol, VLDL/LDL-cholesterol or apo B (see Table 2).

In addition to the degradation of PAF and some lipid parameters also the platelet aggregating behaviour was studied. To eliminate variations due to the medication of patients with

TABLE 2.

Degradation of PAF (nmol/ml x min) in Serum from Survivors of Myocardial Infarction and Healthy Controls Matched for Lipid Parameters

Matched Parameter	N	Controls (Mean±SD)	Patients (Mean±SD)	p
Total cholesterol	19	32.0±7.3	37.7±7.4	**
VLDL/LDL-cholesterol	15	24.5±10	37.0±6.5	**
LDL-cholesterol	18	30.0±7.4	38.6±6.7	**
HDL-cholesterol	15	33.9±8.4	36.7±6.8	n.s.
Triglycerides	20	33.2±8.5	37.8±8.0	n.s.
Apo A-I	15	32.3±11	37.7±6.2	n.s.
Apo B	11	33.1±8.0	40.4±6.4	*

Mean values were compared by the paired WILCOXON rank sum test.
N = number of pairs; n.s. = not significant ($p>0.05$);
*$p<0.05$; **$p<0.01$

TABLE 3

Platelet Aggregation in Plasma from Normal Subjects and Survivors of Myocardial Infarction

Parameter	Controls (N = 36)	Patients (N = 40)	Significance
ADP [ΔT_{1min} (cm)]	1.4±1.2	1.7±1.0	n.s.
ADP [EC_{50} (µmol/l)]	2.5±1.4	2.0±0.6	$p<0.05$
PAF [ΔT_{1min} (cm)]	2.4±1.7	2.7±1.6	n.s.
PAF [EC_{50} (µmol/l)]	0.45±0.4	0.34±0.21	n.s.
Collagen [ΔT_{5min} (cm)]	3.4±1.4	3.7±1.6	n.s.
Spontaneous (%)	14.4±10.7	12.2±8.5	n.s.

Means ± S.D. are shown. N = number of subjects;
n.s. = not significant ($p>0.05$);

ASA, these studies were performed with the use of ASA-treated platelets. Spontaneous aggregation in whole blood as well as the ADP-, PAF-, and collagen-induced aggregation responses were measured in PRP. As shown in Table 3, a statistically significant difference was only obtained by comparing the EC_{50} values of the ADP-induced platelet aggregation.

Linear correlation analysis of the measured parameters revealed a series of significant relationships between the degradation of PAF and the lipoprotein profil (see Table 4). Thus, PAF-hydrolysis in the control group correlated positively with the concentrations of total cholesterol, VLDL/LDL-cholesterol, LDL-cholesterol, as well as apo B. There was also an inverse correlation between the level of HDL-cholesterol and the degradation of PAF. Similar but distinctly weaker relationships were found in the patients group. In contrast, there were no statistically significant correlations between any of the platelet function values and the degradation of PAF as well as any lipid parameter.

TABLE 4

Relationships Between the Degradation of PAF and Concentrations of Lipids and Apolipoproteins

Lipid parameter	Linear correlation coefficient	
	Controls (N = 36)	Patients (N = 40)
Total cholesterol	0.6652***	0.3353*
HDL-cholesterol	-0.4829**	-0.2857n.s.
VLDL/LDL-cholesterol	0.7185***	0.4487**
LDL-cholesterol	0.6891***	0.2750n.s.
Triglycerides	0.3751*	0.2531n.s.
Apo A-I	-0.2233n.s.	-0.1943n.s.
Apo B	0.4924**	0.2954n.s.

n.s. - not significant ($p>0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$
N - number of subjects

Besides single parameters also some ratios have been calculated for each subject (see Table 5). The well established quotients total-/HDL-cholesterol and apo B/apo A-I as well as the ratios PAF-degradation/HDL-cholesterol and PAF-degradation/apo A-I were found to be significantly increased in the

patients group. Using the ratio PAF-degradation/HDL-cholesterol as discriminator, more than 73% of the subjects were classified correctly.

TABLE 5

Means, Cut-off Points and Apparent Error Rates of Several Ratios of Variables

Ratio	Controls (Mean±SD)	Patients (Mean±SD)	COP	ARR (%)	p
Total cholesterol / HDL-cholesterol	4.68±1.5	5.85±1.2	4.90	22	***
Apo B / Apo A-I	0.86±0.2	1.1±0.2	0.85	25	***
Degradation of PAF / HDL-cholesterol	23.2±9.2	34.4±10	28.0	26	****
Degradation of PAF / Apo A-I	0.28±0.1	0.38±0.1	0.32	28	***

COP = cut off point; ARR = apparent error rate; ***p<0.0001; ****p<0.00001

DISCUSSION

It is generally accepted that alterations in serum lipid and lipoprotein values are correlated with atherosclerotic diseases [22]. Increased serum concentrations of total cholesterol, LDL-cholesterol, high blood triglycerides, and reduced levels of HDL are considered as important risk factors for cardiovascular diseases [14]. The present study demonstrates a series of strong relationships between serum lipoproteins, degradation of PAF and the manifestation of coronary artery diseases.

Considering the suggested role of PAF in the development of atherosclerosis [23] it seems quite surprising that the serum capacity to inactivate this highly proinflammatory phospholipid is significantly increased in serum of patients suffering from coronary artery diseases. On the other hand, the degradation

of PAF is catalyzed by a specific acetylhydrolase [5] which is associated with various lipoprotein particles, in particular those containing the apo B [7,8]. In accordance with a previous study [7] a close relationship between the concentrations of lipids and apolipoproteins and the capacity to degrade PAF was found in the control group. At present we have no rational explanation for the markedly weaker relationships found in the patients group. But there is experimental evidence that the degradation of PAF depends not only on the amount of PAF-acetylhydrolase but also on its distribution between the various lipoprotein classes [10]. Therefore, it seems probable that the increased degradation of PAF in serum from myocardial infarction survivors is attributable to differences in the composition of the lipoprotein particles. Such changes might influence the incorporation of PAF and/or the distribution of PAF-acetylhydrolase which are both known to affect the degradation of PAF in serum [10]. Although there is no proof that the relationships between the degradation of PAF in serum and atherosclerosis are causative in nature there are some hints to a possible use of serum PAF-acetylhydrolase as a risk indicator of atherosclerosis.

Using the PAF-degrading capacity of serum as an univariate discriminator, it is proved to exert effects comparably in magnitude to those of the more commonly recognized factors of total cholesterol, HDL-cholesterol and the apo's A-I and B. This finding is supported also by two other studies including patients suffering from peripheral vascular diseases [11] and diabetes mellitus (unpublished results). The group means of the degradation of PAF in serum were also significantly different by comparing subgroups which were matched for plasma levels of total-cholesterol, VLDL/LDL-cholesterol or apo B. Moreover, the quotient PAF-degradation/HDL-cholesterol was identified as a good discriminator. These results point to an additional improvement for the discrimination between low and high risk groups by measuring the degradation of PAF in serum. In spite of these results a final valuation of the predictive value of PAF degradation in serum can be deduced only from a prospective clinical trial.

Although platelet hyperreactivity is considered to play an important role in atherogenesis [24] we did only find a significant difference between patients and controls by comparing the EC_{50} values of the ADP-induced platelet aggregation. Moreover, there were also no significant correlations between serum lipid concentrations and any of the platelet function values in both groups of subjects. There are conflicting reports with respect to altered platelet function associated with coronary artery disease [25,26] as well as correlations between serum lipid levels and platelet reactivity [27,28]. The reason for the discrepant findings may be related partly to methodical differences, or differences in the subject population groups. Considering our results, however, it has to be taken into account that the platelet studies have been performed after blocking the cyclooxygenase pathway by ASA which causes an interruption of the feedback amplification in platelet activation by prostaglandin and thromboxane synthesis [29]. Additionally, the results of our study may be influenced

by platelet inhibitory effects of the calcium channel blockers and the nitrovasodilators [30] taken by some patients.

There was also no evidence for a relationship between the platelet aggregation response towards PAF and its degradation in serum, suggesting that the interaction of PAF with platelets in plasma [31] is not regulated by the PAF-degrading enzyme the PAF-acetylhydrolase.

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REFERENCES

[1] HANAHAN, D.J.: Platelet-activating factor: A biologically active phosphoglyceride. Ann. Rev. Biochem., 55, 483-509, 1986.

[2] McMANUS, L.M.: Pathobiology of platelet-activating factors. Pathol. Immunopathol. Res., 5, 104-117, 1986.

[3] BLANK, M.L., CRESS, E.A., WHITTLE, T., and SNYDER, F: In vivo metabolism of a new class of biologically active phospolipids: 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, a platelet activating - hypotensive phospholipid. Life Sci., 29, 769-775, 1981.

[4] BLANK, M.L., LEE, T.-C., FITZGERALD, V., and SNYDER, F.: A specific acetylhydrolase for 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine (a hypotensive and platelet-activating lipid). J. Biol. Chem., 256, 175-178, 1981.

[5] STAFFORINI, D.M., PRESCOTT, S.M., and Mc INTYRE, T.M.: Human plasma platelet-activating factor acetylhydrolase. Purification and properties. J. Biol. Chem., 262, 4223-4230, 1987.

[6] FARR, R.S., WARDLOW, M.L., COX, C.P., MENG, K.E., and GREENE, D.E.; Human serum acid-labile factor is an acetylhydrolase that inactivates platelet-activating factor. Feder. Proc., 42, 3120-3122, 1983.

[7] OSTERMANN, G., KERTSCHER, H.-P., WINKLER, L., SCHLAG, B., RÜHLING, K., and TILL, U.: The role of lipoproteins in the degradation of platelet-activating factor. Thromb. Res., 44, 303-314, 1986.

[8] STAFFORINI, D.M., Mc INTYRE, T.M., CARTER, M.E., and PRESCOTT, S.M.: Human plasma platelet-activating factor acetylhydrolase. Association with lipoprotein particles and role in the degradation of platelet-activating factor. J. Biol. Chem., 262, 4215-4222, 1987.

[9] BLANK, M.L., HALL, M.N., CRESS, H.A., and SNYDER, F.: Inactivation of 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine by a plasma acetylhydrolase: higher activities in hypertensive rats. Biochem. Biophys. Res. Commun., 113, 666-671, 1983.

[10] OSTERMANN, G., KOSTNER, G.M., GRIES, A., MALLE, E., and TILL, U.: The contribution of individual lipoproteins to the degradation of platelet-activating factor (PAF) in human serum. Haemostasis. Submitted for publication.

[11] OSTERMANN, G., RÜHLING, K., ZABEL-LANGHENNIG, R., WINKLER, L., SCHLAG, P., and TILL, U.: Plasma from atherosclerotic patients exerts an increased degradation of platelet-activating factor. Thromb. Res., 47, 279-285, 1987.

[12] PRICHARD, P.H.: The degradation of platelet-activating factor by high-density lipoprotein in rat plasma. Effect of ethynylestradiol administration. Biochem. J., 246, 791-794, 1987.

[13] PRICHARD, P.H., CHONN, A., and YEUNG, C.C.H.: The degradation of platelet-activating factor in the plasma of a patient with familial high density lipoprotein deficiency (Tangier disease). Blood, 65, 1476-1478, 1985.

[14] GRUNDY, S.M., GREENLAND, P., HERD, A., HUEBSCH, J.A., JONES, R.J., MITCHELL, J.H., and SCLART, R.C.: Cardiovascular risk factor evaluation of healthy american adults. Circulation, 75, 1340A-1362A, 1987.

[15] PACKHAM, M.A. and MUSTARD, J.F.: The role of platelets in the development and complications of atherosclerosis. Semin. Hematol., 23, 8-26, 1986.

[16] KERTSCHER, H.-P.: Synthese von rac. 1-O-Hexadecyl-2-O-acetyl-glycero-3-phosphocholin (PAF-acether). Pharmazie, 38, 421, 1983.

[17] ROSE, G.A.: The diagnosis of ischemic heart pain and intermittent claudication in field surveys. Bull. World Health Organ., 27, 645-658, 1962.

[18] FRIEDEWALD, W.J., LEVY, R.I., and FREDERICKSON, D.S.: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of preparative ultracentrifuge. Clin. Chem., 18, 499-503, 1972.

[19] WINKLER, L., SCHLAG, B., RÜGER, H.J., and DARGEL, R.: Ein standardisierungsfähiges Verfahren zur quantitativen Bestimmung von Apolipoprotein A-I im Serum mittels Immunturbidimetrie am Spekol-Photometer. Z. med. Labor.-Diagn., 27, 127-129, 1986.

[20] WINKLER, L., SCHLAG, B., RÜGER, H.J., and DARGEL, R.: Ein standardisierungsfähiges Verfahren zur quantitativen Bestimmung von Apolipoprotein B im Serum mittels Immunturbidimetrie am Spekol-Photometer. Z. med. Labor.-Diagn., 26, 293-295, 1985.

[21] BORN, G.V.R.: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature, 194, 927-929, 1962.

[22] KOSTNER, G.M.: Apolipoproteins and lipoproteins of human plasma: significance in health and in disease. Adv. Lipid Res., 20, 1-43, 1983.

[23] HANDLEY, D.A. and SAUNDERS, R.N.: Platelet activating factor and inflammation in atherogenesis: targets for drug development. Drug Dev. Res., 7, 361-375, 1986.

[24] REES, B.W.G.: Hypothesis supporting the primary role of increased platelet activity in atherosclerosis and coronary heart disease. Med. Lab. Sci., 44, 267-271, 1987.

[25] HEPTINSTALL, S.: Platelet behaviour and vascular disease. Artery, 8, 448-456, 1980.

[26] BREDDIN, K.: Detection of prethrombotic states in patients with atherosclerotic lesions. Semin. Thromb. Hemost., 12, 111-123, 1986.

[27] MEADE, T.W., VICKERS, M.V., THOMPSON, S.G., STIRLING, Y., HAINES, A.P., and MILLER, G.J.: Epidemiological characteristics of platelet aggregability. Br. Med. J., 290, 428-432, 1985.

[28] NIMPF, J., WURM, H., KOSTNER, G.M., and KRONER, Th.: Platelet activation in normo- and hyperlipoproteinemias. Basic Res. Cardiol., 81, 437-453, 1986.

[29] HARKER, L.A. and FUSTER, V.: Pharmacology of platelet inhibitors. J. Am. Coll. Cardiol., 8, 21B-32B, 1986.

[30] FUSTER, V., ADAMS, P.C., BADIMON, J.J., and CHESEBRO, J.H.: Platelet-inhibitor drugs' role in coronary artery disease. Progr. Cardiovasc. Dis., 29, 325-346, 1987.

[31] OSTERMANN, G., TILL, U., and THIELMANN, K.: Studies on the stimulation of human blood platelets by semisynthetic platelet-activating factor. Thromb. Res., 30, 127-136, 1983.

EXHIBIT 5

Lupus (1994) 3, 55–58

Antibodies Against Platelet-Activating Factor in Patients with Antiphospholipid Antibodies

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We have studied the specificity of antiphospholipid antibodies in 148 patients with autoimmune diseases, 120 patients with systemic lupus erythematosus and 28 with the primary antiphospholipid syndrome. In addition, 20 patients suffering from syphilis were studied. As a control group, 64 healthy volunteers were investigated. Patient and control serum samples were tested for binding to seven different phospholipid antigens by ELISA. Interestingly, 90% of the sera from syphilis patients and 6% of the autoimmune patients exhibited a significant binding to platelet-activating factor (PAF), a molecule similar to the structure of phosphatidylcholine. In addition, the IgG fraction from one of the lupus patients, which showed a high binding activity to PAF, was further affinity-purified using both liposomes and an affinity chromatography column. Preincubation of these antibodies with PAF inhibited subsequent binding to immobilized PAF. These observations might suggest a putative interaction of antiphospholipid autoantibodies with PAF '*in vivo*', which may have, in some patients, important pathophysiological consequences.

Key Words: Thrombosis Syphilis Antiphospholipid Platelet activating factor SLE

Introduction

Antiphospholipid antibodies from patients with autoimmune diseases such as systemic lupus erythematosus (SLE) or the recently described primary antiphospholipid syndrome (PAPS)[1,2] detected with immunoenzymatic methods, usually react only with negatively charged phospholipids such as cardiolipin (CL), phosphatidic acid (PA), phosphatidylinositol (PI) or phosphatidylserine (PS)[3,4]. Reactivity against zwitterionic phospholipids was reported in some patients[5]. Platelet-activating factor (PAF) is an ether-phospholipid with a molecular structure very similar to phosphatidylcholine; it has many biological functions[6]. In the early 1970s, this substance was initially found to be released by basophils during IgE-induced anaphylaxis[7,8] and subsequently also in patients with SLE[9]. It was identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine[10,11]. It was also shown that many other types of cells could release PAF under certain situations. These include platelets, endothelial cells, neutrophils and macrophages[12,13].

The immunogenicity of PAF was first reported by Nishihira *et al.* in 1984[14]. These authors produced antibodies that reacted *in vitro* against PAF by immunization of a mouse with this phospholipid. Later, other researchers confirmed the ability of PAF to induce a specific antibody response[15–17]. An immunoassay for the measurement of PAF levels was developed by using specific antibodies[18]. More recently, the fine specificity of anti-PAF antibodies raised in immunized rabbits was studied by Cooney *et al.*[19]

Herein we report the results of our search for antibodies against PAF in patients with SLE, PAPS, syphilis and in normal blood donors. To study its binding specificities, we also affinity-purified the plasma sample with the highest anti-PAF activity in the ELISA by using two different methods: a liposome-based technique and a chromatography column coated with PAF.

Patients and methods

Patients

One hundred and twenty patients who fulfilled the American Rheumatism Association criteria for the classification of SLE[20], 28 patients with PAPS, 40 patients with syphilis and 64 normal subject (20 blood donors and 44 healthy hospital workers) were included in this study. Plasma samples were obtained by centrifugation (3000 rpm) of citrated blood for 20 min, aliquoted and stored at −20°C until ready for use. The patient whose plasma was chosen for the anti-PAF purification was a 70-year-old woman who had suffered from SLE since 1956 and who had the following manifestations: polyarthritis, five unexplained fetal losses, thrombocytopenia, positive tests for antinuclear and anti-DNA antibodies, positive lupus anticoagulant test and IgM anticardiolipin (20 MPL).

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ELISA technique for anti-PAF

The ELISA method used to determine the presence of anticardiolipin (aCL) antibodies[21] was modified to detect antibodies against PAF. Briefly, 30 µl of PAF (Sigma) (50 µg/ml) dissolved in methanol/chloroform (3:1) were added to each well in a microtiter plate and left overnight at 4°C. The next steps were similar to those in the aCL ELISA and have been described elsewhere[21]. Positive reactions were compared with those obtained with the aCL immunoassay and quantification was done using the international units (GPL and MPL) used for aCL antibodies[21]. Non-specific binding was ruled out in all positive samples by running them in empty ELISA plates.

Affinity purification of anti-PAF antibody

Purification utilizing PAF liposomes Three ml of the patient's serum with the highest activity against PAF were mixed with 5 mg/ml PAF micelles prepared as described elsewhere[22] and incubated at 4°C overnight. On the following day this mixture was centrifuged for 1 h at 15 000 rpm and the precipitate separated. This precipitate was washed with phosphate buffered saline (PBS) and the suspension centrifuged again at 15 000 rpm for 1 h. This process was repeated twice. After the third wash the precipitate was resuspended in 1.5 M sodium iodide (NaI), vortex mixed and left to stand for 15 min. An equal volume of chloroform was then added, vortex mixed and allowed to stand and centrifuged again at 3000 rpm for 10 min. The aqueous layer that contained the affinity purified antibody was separated and dialysed against PBS overnight.

Chromatography column coated with PAF PAF (10 mg) (Sigma Chemical Co., St. Louis, MO) was mixed with 50 mg cholesterol (Sigma Chemical Co.) in a glass scintillation vial and evaporated under nitrogen as described elsewhere[23]. Ethanol (1 ml) was added and the vial was capped, placed in boiling water and swirled until the lipids were dispersed. The vials were then removed and, after cooling, a 10 ml solution of 15% acrylamide, 5% BIS acrylamide (BIO RAD, Cambridge, MA) was added, followed immediately by addition of 100 µl of ammonium persulfate (140 mg/ml) and 5 µl of TEMED. The mixture was transferred to a glass test tube, covered with parafilm and aluminum foil and allowed to polymerize overnight at 4°C. The rigid white gel was removed from the tube, rinsed with distilled water and minced with a razor blade. The gel was then homogenized using a hand operated loose fitting teflon pestle. The homogenized gel was washed three times in distilled water, allowing the gel to settle for 10 min and decanting the supernatant on each occasion. The settled gel particles were then assembled into a column (125 × 20 mm) and equilibrated with eight to ten bed volumes of PBS (0.01 M phosphate/0.15 M NaCl buffer), pH 7.3. Flow rates of 50–60 ml/h were used with only moderate compaction of the relatively rigid gel particles. Elution of affinity purified immunoglobulin was performed according to the following protocol. After equilibration, 8 ml of patient plasma diluted 1:4 in PBS at the same rate until absorbance of fractions at 280 nm was <0.01 absorbance units. Then 30 ml of eluting buffer, 0.1 M phosphate/ 0.5 M NaCl buffer, pH 7.3 were applied to the column at 40–50 ml/h. The eluate was collected in 2 ml fractions and optical density readings and anti-PAF activity (ELISA) were determined. These anti-PAF antibodies were tested against negatively charged and zwitterionic phospholipids. Some fractions were freeze-dried and reconstituted with smaller volumes of distilled water as a means of concentrating them for lupus anticoagulant testing.

Inhibition studies

Inhibition studies of the affinity-purified IgM anti-PAF antibody were performed by using a previously described method[24]. In brief, known amounts of the affinity-purified anti-PAF antibody diluted in PBS were incubated at 37°C overnight with increasing concentrations of PAF that ranged from 0.125 to 1 mg/ml. Different dilutions of these mixtures were then tested in the ELISA assay against PAF.

Coagulation studies

The lupus anticoagulant (LA) activity was measured by the ability of 0.1 ml of the affinity-purified IgM anti-PAF to prolong the diluted tissue thromboplastin time when mixed with 0.1 ml of normal plasma compared with the mixture of 0.1 ml of this plasma with 0.1 ml of Tris buffer, both measured after incubation at 37°C for 5 min.

Results

Anti-PAF activities of plasma samples

Syphilis patients displayed an average binding activity higher than the mean of the normal plus five standard deviations (SD), this difference being highly significant

Table 1 Number of anti-PAF antibody-positive patients in the different groups.

	SLE (*n* = 120)	*PAPS* (*n* = 28)	*Syphilis* (*n* = 40)	*Controls* (*n* = 64)
Anti-PAF IgG	4	3	14	0
Anti-PAF IgM	5	1	12	1
Anti-PAF IgG + IgM	1	1	4	0

Samples with >5 GPAFL and/or MPAFL units were considered positives.

MPAFL/GPAFL

80
60
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Figure 1 of patient lupus ery (+) IgG.

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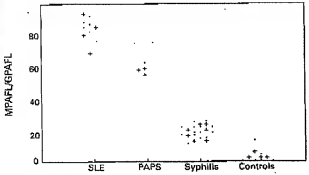

Figure 1 Distribution of anti-PAF antibody levels in the different groups of patients. MPAFL, units for IgM; GPAFL, units for IgG; SLE, systemic lupus erythematosus; PAPS, primary antiphospholipid syndrome. (•) IgM; (+) IgG.

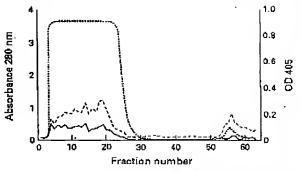

Figure 2 Column chromatography affinity-purified anti-PAF antibodies. OD, optical density. (——) aCL activity, (·····) protein concentration, (– – –) aPAF activity.

compared with the control group ($P < 0.01$). The differences between these groups and SLE and PAPS groups were not statistically significant, probably because of the heterogeneity in the latter two groups. However, 10 samples in the SLE group and five in the PAPS group showed high binding in the ELISA plates (Table I). We compared the GPL and MPL international units[22] with our optical density and created the PAF units (GPAFL and MPAFL).

In the group of normal blood donors, only one plasma demonstrated low IgM anti-PAF activity in the ELISA. Distribution of the anti-PAF antibodies levels of the four groups of patients are represented in Figure 1.

Affinity purified anti-PAF

Affinity purified anti-PAF had anti-PAF activity of 20 MPAFL. When tested by an ELISA method against negatively charged phospholipids (PS, PA, PI, CL) and against zwitterionic phospholipids (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine) this showed no crossreactivity. There were no differences between liposomes and chromatography column in the affinity purified anti-PAF.

Inhibition studies

The affinity-purified IgM anti-PAF antibody when mixed with increasing concentrations of PAF was progressively inhibited. Other phospholipids such as CL or phosphatidylcholine were able to inhibit PAF binding activity, although PAF produced the highest inhibition compared with that achieved by two other phospholipids (data not shown).

Coagulation studies

Affinity-purified IgM anti-PAF antibody did not prolong the diluted tissue thromboplastin time when mixed with normal plasma compared with the mixture of this plasma with Tris buffer.

Inhibition and coagulation studies with the anti-PAF obtained with chromatography column did not differ from the liposomes affinity purified anti-PAF. The anti-PAF activity of the purified fraction was moderate (20 MPAFL) and similar to the aCL activity of the serum (Figure 2.).

Discussion

In our study, most plasma samples from patients with syphilis had low levels of antibodies that bound to PAF *in vitro* when compared with those from normal blood donors. Sera from patients with autoimmune diseases also showed significant binding. Two of these patients had high serum levels. Only differences between syphilis and normal blood donors were significant ($P < 0.01$).

Six of eight patients with autoimmune diseases (SLE and PAPS) that reached high positive values in the assay showed strong non-specific binding when tested in a plate without antigen. Only two sera showed high specific binding to PAF (IgM class). One of these patients had SLE with thrombotic manifestations and the other had autoimmune thrombocytopenic purpura. Both patients also had IgM aCL. The anti-PAF activity, the absence of crossreactivity with other phospholipids and the results of the inhibition studies performed with the affinity-purified antibody demonstrated that at least some of the antibodies against PAF may be specific and exist in some autoimmune and infectious conditions.

Although PAF binding specificities were previously reported by us and by other authors[25–27], our study was the first one that specifically studied these antibodies in various human diseases.

As most authors agree that antiphospholipid antibodies are heterogeneous, anti-PAF antibodies might represent a new specificity within this large family of autoantibodies.

A recent study showed that PAF may activate thrombolysis in response to soluble aggregates of immunoglobulin G[28]. Antibodies that block the action of PAF could then inhibit fibrinolysis and promote thrombosis[29].

Acknowledgements

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References

1. Harris EN. Syndrome of the black swan. *Br J Rheumatol* 1987; 26: 324–6.
2. Asherson RA, Khamashta MA, Ordi-Ros J *et al.* The 'primary' antiphospholipid syndrome: major clinical and serological features. *Medicine* (Baltimore) 1989; 68: 366–74.
3. Harris EN, Gharavi AE, Wasley GD. Hughes GRV. Use of an enzyme-linked immunosorbent assay and of inhibition studies to distinguish between antibodies to cardiolipin from patients with syphilis or autoimmune diseases. *J Infect Dis* 1988; 157: 23–31.
4. Gharavi AE, Harris EN, Asherson RA, Hughes GRV. Anticardiolipin antibodies: isotype distribution and phospholipid specificity. *Ann Rheum Dis* 1987; 46: 1–6.
5. Staub HL, Harris EN, Khamashta MA, Savidge G, Chahade WH, Hughes GRV. Antibody to phosphatidylethanolamine in a patient with lupus anticoagulant and thrombosis. *Ann Rheum Dis* 1989; 48: 166–9.
6. Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. *Pharmacol Rev* 1987; 39: 97–145.
7. Benveniste J, Henson PM, Cochrane CG. Leukocyte-dependent, histamine release from rabbit platelets: the role of IgE, basophils and a platelet-activating factor. *J Exp Med* 1972; 136: 1356.
8. Pinckard RN, Farr RS, Hanahan DJ. Physicochemical and functional identity of rabbit platelet-activating factor (PAF) released *in vivo* during IgE anaphylaxis with PAF released *in vitro* from IgE sensitized basophils. *J Immunol* 1979; 123: 1847.
9. Camussi G, Tetta C, Coda R, Benveniste J. Release of platelet-activating factor in human pathology. I. Evidence for the occurrence of basophil degranulation and release of platelet-activating factor in systemic lupus erythematosus. *Lab Invest* 1981; 44: 241–51.
10. Benveniste J, Tence M, Bidault J, Boullet C, Polonsky J. Semisynthèse et structure proposée du facteur activant les plaquettes (PAF). *C R Acad Sci (D) (Paris)* 1979; 289: 1037.
11. Demopoulos CA, Pinckard RN, Hanahan DJ. Platelet-activating factor: evidence for 1-O-alkyl-2-*sn*-glycerll-3-phosphoryl-choline as the active component. (A new class of lipid chemical mediators.) *J Biol Chem* 1979; 254: 9255.
12. Camussi G, Mencia-Huerta JM, Benveniste J. Release of platelet-activating factor and histamine. I. Effect of immune complexes, complement and neutrophils on human and rabbit mastocytes and basophils. *Immunology* 1977; 33: 523.
13. Lotner GZ, Lynch IM, Betz S, Henson PM. Human neutrophil-derived platelet-activating factor (PAF). *J Immunol* 1980; 124: 676.
14. Nishihira J, Ishibashi T, Imai Y. Production and characterization of specific antibodies against 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (a potent hypotensive and platelet-activating ether-linked phospholipid). *J Biochem (Tokyo)* 1984; 95: 1247–51.
15. Karasawa K, Fujita K, Satoh N *et al.* Antibody to platelet activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF). *J Biochem (Tokyo)* 1987; 102: 451–3.
16. Small MA, Baldo BA, Redmond JW. Production of antibodies against platelet activating factor. *Mol Immunol* 1989; 26: 711–19.
17. Macpherson JL, Spur B, Pyne SG *et al.* Production and characterization of antibodies to platelet activating factor. *J Lipid Mediat* 1992; 5: 49–59.
18. Small MA, Baldo BA, McCaskill A. A specific, sensitive radioimmunoassay for platelet-activating factor (PAF). *J Immunol Methods* 1990; 128: 183–8.
19. Cooney SI, Small MA, Hirle DG, Baldo BA. Combining site specificities of rabbit antibodies to platelet-activating factor (PAF). *Mol Immunol* 1990; 27: 405–12.
20. Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271–7.
21. Loizou S, McCrea JD, Rudge AC, Reynolds R, Boyle CC, Harris EN. Measurement of anticardiolipin antibodies by an enzyme-linked-immunosorbent assay (ELISA): standardization and quantitation of results. *Clin Exp Immunol* 1985; 62: 738–45.
22. Harris EN, Gharavi AE, Tincani A *et al.* Affinity-purified anticardiolipin and anti-DNA antibodies. *J Clin Lab Immunol* 1985; 17: 155–62.
23. Harris EN. Annotation: antiphospholipid antibodies. *Br J Haematol* 1989; 73: 139–42.
24. McNeil HP, Krilis SA, Chesterman CN. Purification of antiphospholipid antibodies using a new affinity method. *Thromb Res* 1988; 52: 641–6.
25. Loizou S, Mackworth-Young C, Cofiner C, Walport MJ. Heterogeneity of binding reactivity to different phospholipids of antibodies from patients with SLE and syphilis. *Clin Exp Immunol* 1990; 7: 67–74.
26. Barquinero J, Ordi J, Molina A, Tolosa C, Selva A, Vilardell M. Antibodies against platelet activating factor in patients with antiphospholipid antibodies. *Seventh International Congress of Immunology*, Berlin, 1989.
27. Barquinero J, Selva A, Ordi J, Molina A, Tolosa C, Vilardell M. Anti-PAF antibodies in patients with systemic lupus erythematosus, 'primary' antiphospholipid syndrome, progressive systemic sclerosis and syphilis and its correlation with other antiphospholipid antibodies. *Br J Rheumatol* 1990; 24: 106 (abstract).
28. Fernández-Gallardo S, Gijón MA, Cano E, Sánchez Crespo M. Platelet-activating factor mediates thrombolysis but no thrombocytopenia in response to soluble aggregates of immunoglobulin G. In: *The Role of Platelet-Activating Factor In Immune Disorders. New Trends in Lipid Mediators Research.* Braquet P (ed.). Basel, Karger, 1988; 2: 199–203.
29. Braquet P, Bourgain R, Mencia-Huerta JM. Effect of platelet-activating factor on platelets and vascular endothelium. *Semin Thromb Haemost* 1989; 15: 184–92.

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EXHIBIT 6

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A specific, sensitive radioimmunoassay for platelet-activating factor (PAF)

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A specific radioimmunoassay (RIA) has been developed for platelet-activating factor (PAF) and shown to be sensitive over the range 10–1000 pg (0.02–2 pmol). The anti-PAF antibodies showed specificity for the acetyl group at the C2 position of the PAF molecule and exhibited no significant cross-reactivity with lyso-PAF or the naturally occurring lipids including lecithin and lysolecithin. The sensitivity of the RIA was at least as good as the platelet-based assays for PAF but the RIA was simpler to perform, had a higher capacity and did not have the drawback of the inherent variability associated with the bioassays.

Key words: Platelet-activating factor radioimmunoassay; Anti-platelet-activating factor; Platelet-activating factor; Quantitation of platelet-activating factor

Introduction

Platelet-activating factor (PAF) (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (Hanahan et al., 1980) is a potent biologically active phospholipid which induces platelet aggregation at concentrations as low as 0.1 nM. The biological actions of PAF are diverse and well-documented (Hanahan, 1986). Many cells and tissues are capable of synthesizing and releasing PAF in response to specific stimuli (Snyder, 1985; Barnes et al., 1988). Because of its potency and diverse bioactions, PAF has been implicated in many diseases including asthma, anaphylaxis, allergy, septic shock, gastrointestinal ulceration, acute graft rejection and certain kidney disorders (Braquet et al., 1987; Vargaftig and Braquet, 1987). However, unequivocal conclusions regarding the physiological role of PAF remain difficult whilst there are no precise assays available for its quantitation.

Routine and precise quantitation of low levels of PAF in large numbers of samples by standard physicochemical techniques (Hanahan and Kumar, 1987) is not a practical proposition. The most common methods used at the present time for the detection and measurement of PAF rely on the interaction of the mediator with platelets and measuring either the resultant aggregation or the release of tritiated serotonin (Hanahan and Weintraub, 1985). Although these methods are sensitive, they are not easy to perform and suffer from the inherent variability common to all bioas-

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Abbreviations: PAF, platelet activating factor (1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine); RIA, radioimmunoassay; NSS, normal sheep serum; AcT, 0.05 M sodium acetate buffer pH 6.0 containing 0.05% Tween 20 and 0.1% sodium azide; NSB, non-specific binding; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine.

says. Moreover, in biological samples there may be substances other than PAF that induce activation of platelets and other substances that inhibit the action of PAF on platelets. Consequently, it has been necessary to purify samples by chromatography before analysis by bioassay (Hanahan and Weintraub, 1985).

A simple and specific immunoassay for PAF should overcome most of these problems and permit both routine and accurate quantitation of this important lipid in large numbers of samples. Recently, anti-PAF antibodies with the required specificity have been produced in our laboratories (Smal et al., 1989) and have now been used in the development of simple PAF-specific radioimmunoassay (RIA) of the required sensitivity.

Materials and methods

Materials

C_{16}-PAF, C_{18}-PAF and C_{18}-dehydro-PAF were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Polyethylene glycol MW 6000 and organic solvents were purchased from BDH Chemicals (Kilsyth, Vic., Australia) and Ajax Chemicals (Sydney, Australia) respectively. 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine was a gift from Dr A. Tokumura (University of Tokushima, Japan). All other lipids were purchased from the Sigma Chemical Co. (St. Louis, MO). The IgG fraction of donkey anti-sheep antiserum was obtained from Silenus Laboratories, Melbourne, Australia. ^{125}I-labelled PAF was a kind gift from Dr J. Czarnecki, Silenus Laboratories.

Antisera

C_{12}-PAF-methylated BSA was prepared as previously described (Smal et al., 1989). Sheep received intramuscular injections of 1 mg of this antigen emulsified in Freund's complete adjuvant. 1 month and 4 months after priming, the sheep were boosted with 1 mg of antigen in Freund's incomplete adjuvant and the animals were bled 11 days after the last immunization. PAF-acetylhydrolase activity in the antiserum was destroyed by incubating serum (1 vol.) with 1 M acetic acid (1 vol.) for 6 h before adding 0.2 M phosphate buffer pH 7.2 (8 vols.). This solution was supplemented with similarly deactivated normal sheep serum (NSS).

Titre determination

Initial experiments determined the optimal dilutions of donkey anti-sheep Ig (Silenus Laboratories) and NSS required to give maximal precipitation of ^{125}I-PAF for a given level of anti-PAF. Anti-PAF antisera were titrated in 0.05 M sodium acetate pH 6.0 buffer containing 0.05% Tween 20 and 0.1% sodium azide (AcT) and supplemented with NSS to give a fixed level of sheep serum. The diluted sera were assayed as described below, and the titres taken as the dilutions of the anti-PAF anti-sera which precipitated 40–50% of the total ^{125}I-PAF.

Extraction of saliva

This was carried out according to the procedure of Bligh and Dyer (1959). Saliva (0.8 ml) was mixed with chloroform (1.0 ml) and methanol (2.0 ml) and the mixture sonicated and vortexed extensively. Water (1.0 ml) and chloroform (1.0 ml) were added and, after vortexing, the mixture was centrifuged to achieve separation of the two phases. The lower chloroform phase was evaporated and the residue reconstituted in AcT buffer (0.8 ml).

Radioimmunoassay procedure

A PAF standard solution (0.1 mg/ml in aqueous ethanol, consisting of equal parts C_{16}-PAF and C_{18}-PAF) was diluted in AcT buffer to give standard solutions over the range 0.1–25 ng/ml. Acid-treated anti-PAF antiserum was used at a dilution of 1/8000 in acid-treated NSS 1/2000 in AcT. The donkey anti-sheep Ig was diluted 1 in 250 in AcT buffer containing 6% polyethylene glycol and ^{125}I-PAF (2200 Ci/mmol, DuPont-NEN (NEK-062), Boston, MA) was added to give approx. 40,000 cpm per 100 µl. Into duplicate polystyrene RIA tubes (Disposable Products, Australia) were placed 100 µl of each of the following: sample or PAF standard solution, anti-PAF antiserum, and anti-sheep Ig/tracer. The B_0 tubes contained no PAF, and the non-specific binding (NSB) tubes contained only the anti-sheep Ig and tracer solutions, with AcT buffer added in place of sample and anti-PAF. The tubes were

incubated at room temperature for 16 h, 4 ml AcT was added and the tubes were centrifuged at 1900 $\times g$ for 25 min. After decanting the supernatants, the radioactivity remaining in the tubes was measured and the percent of tracer bound to the precipitate (% B/B_0) calculated from the formula $(B - \text{NSB})/(B_0 - \text{NSB}) \times 100$. The amount of PAF in the samples was determined from the standard curve obtained by plotting PAF concentration against % B/B_0.

Inhibition studies

Solutions of some commonly occurring lipids and selected PAF analogues were formulated in AcT buffer and were then tested in the RIA, replacing the PAF standard solutions. The effects of these compounds on the assay were calculated as % B/B_0.

Results

Radioimmunoassay performance

The percent of tracer bound to the antibody in the absence of PAF (B_0) ranged from 35 to 45%. However, as the labelled PAF aged, this gradually dropped to below 30%. Non-specific binding (NSB) was low, ranging from 1 to 2%.

Since natural PAF is a mixture of various alkyl analogues, with C_{16} and C_{18} analogues generally predominating, the standard chosen for the RIA was an equimolar mixture of these two analogues. Bound ^{125}I-PAF could be displaced from the antibody complex with increasing concentrations of standard PAF, generating a standard curve as shown in Fig. 1. The curve, which was linear over the range 0.5–10 ng/ml (50–1000 pg per tube), could be used to quantitate PAF from 25 pg (0.05 pmol) to 2500 pg (5 pmol) per tube. Sensitivities down to 9 pg/tube were obtained in the present studies. In four separate assays using the same batch of tracer, the values for 50% inhibition were 1.30, 1.40, 1.65 and 1.70 ng/ml. Generally, as the age of the tracer increased, these values also increased.

Specificity

The specificity of anti-PAF antibodies was determined by quantitative hapten inhibition studies

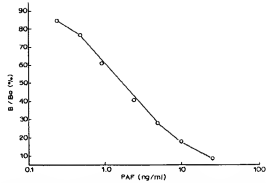

Fig. 1. A typical standard curve obtained for the PAF RIA using an equimolar mixture of C_{16}- and C_{18}-PAF together with sheep anti-PAF and ^{125}I-PAF.

using selected analogues of PAF in the RIA. The results are shown in Fig. 2. C_{16}-PAF proved to be the most reactive analogue, requiring 0.39 pmol for 50% displacement, whereas 0.48 pmol of C_{18}-dehydro-PAF and 0.72 pmol of C_{18}-PAF were required for 50% inhibition.

The acyl analogue, 1-palmitoyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (1-palmitoyl-AGPC), was poorly recognised by the antibodies and 87 pmol of this compound were required to achieve 33% inhibition.

The commonly occurring lipids were also tested for their potential to inhibit the assay (Table I) at concentrations up to 20 μg/ml. No significant inhibition was observed with any of these compounds.

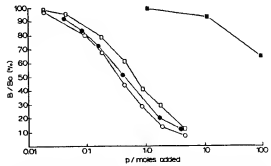

Fig. 2. Recognition of PAF analogues by sheep anti-PAF in the PAF RIA. Inhibition curves obtained with C_{16}-PAF (○), $C_{18:1}$ (dehydro)-PAF (●), C_{18}-PAF (□) and 1-palmitoyl-AGPC (■).

TABLE I

SPECIFICITY OF THE PAF RIA: EFFECTS OF COMMON LIPIDS EXAMINED FOR INHIBITORY ACTIVITY

Lipid	B/B_0 (%) at	
	20 μg/ml	4 μg/ml
Cholesteryl oleate	102	104
Triolein	101	102
Cholesterol	96	99
Oleic acid	100	100
Phosphatidyl ethanolamine	96	104
Phosphatidyl serine	103	106
Phosphatidyl inositol	106	102
Phosphatidyl choline	101	103
Sphingomyelin	99	103
Lyso-phosphatidyl ethanolamine	102	105
Lyso-phosphatidyl choline	96	103
AcT buffer only	100	100

Lyso-PAF, the primary metabolite of PAF, was tested for inhibitory potency at high and low concentrations. At less than 100 ng/ml no inhibition was observed. At very high concentrations (0.8–100 μg/ml), some displacement occurred. Cross-reactivity with the standard PAF mixture (inhibition within the range of 0.1–10 ng/ml) was 1 in 40,000.

Measurement of PAF in saliva by RIA

The PAF level in a sample of human saliva was quantitated by RIA. Lipids were extracted using chloroform-methanol-water (Bligh and Dyer, 1959) in order to eliminate any effects due to the adsorption of PAF by particulate matter in saliva (Smal, Roche, Cooney and Baldo, unpublished).

TABLE II

QUANTITATION OF PAF IN HUMAN SALIVA EXTRACT [a] BY RIA

Dilution of saliva	PAF concentration (ng/ml) [b]	
	No added PAF	With added PAF 2.5 ng/ml
1 in 2	1.6	4.7
1 in 4	0.83	3.3
1 in 8	0.35	3.0

[a] Bligh-Dyer (chloroform-methanol-water) extract.
[b] Average of duplicates

The extracts were then tested at different dilutions and with added PAF in order to determine whether the assay was correctly determining PAF levels and whether the lipids caused any interference. The PAF levels recorded were found to be within the expected range (Table II).

Discussion

In an earlier study we demonstrated that rabbit antibodies to PAF could be produced following the injection of a C_{12}-PAF analogue conjugated to methylated BSA (Smal et al., 1989). These polyclonal antibodies had the required specificity and initial attempts to develop a radioimmunoassay resulted in an assay similar to the one described here but with a sensitivity of 1 ng/ml. In an attempt to improve the sensitivity and ensure a continuity of supply of the antiserum, the PAF immunogen was injected into sheep. This resulted in the production of high titre, PAF-specific antisera suitable for RIA use.

The procedure for the present assay is straight forward, requiring only the addition of four components. The PAF-anti-PAF complex is precipitated with a second-antibody and polyethylene glycol facilitates this. Moreover, the use of the gamma emitter ^{125}I-PAF as the trace results in excellent sensitivity; alternatively ^{3}H-PAF can be used instead but this leads to diminished sensitivity (results not shown).

It has been shown that natural PAF is not a single molecular species, but a mixture of alkyl analogues, commonly C_{18}-PAF, C_{16}-PAF and C_{18}-dehydro-PAF (Mueller et al., 1984; Mallet and Cunningham, 1985; Ramesha and Pickett, 1987). Recognition of these compounds by the antibodies was similar, although not identical. The PAF standard chosen for use in the RIA was an equimolar mixture of the C_{16}- and C_{18}-PAF since this combination is probably adequate for most practical purposes.

Once the distribution in tissues and fluids of the biologically similar but structurally different PAFs has been determined, it may become necessary in the future to use a specific combination of the different analogues when measuring PAF from a specific source. It has also been demonstrated

that acyl PAF analogues may be produced concomitantly with alkyl PAF (Mueller et al., 1984; Satouchi et al., 1985; Tokumura et al., 1987) although the ratio of these two analogues is variable (ranging from 1:1 to 100:1) depending on the source. In the RIA, cross-reactivity with the acyl PAF is less than 1/500, so very little acyl PAF is likely to be detected.

The primary metabolite of PAF is lyso-PAF, which lacks the acetyl group and frequently occurs in tissues and fluids in much larger quantities than PAF (Pettipher et al., 1987; Prevost et al., 1988). The interaction of this substance with the anti-PAF antibodies is extremely weak and hence no cross-reactivity problems with lyso-PAF are envisaged. Further inhibition studies aimed at mapping the antibody combining sites in great detail have shown that there is a specific requirement for a short chain acyl group, particularly acetyl, at carbon-2 of the glycerol skeleton (Smal, Baldo and Harle, manuscript in preparation).

Since most extracts of biological samples are likely to contain large quantities of commonly occurring natural lipids, such as cholesterol, phosphatidyl choline, lyso-phosphatidyl choline etc. (see Table I), these substances were also tested in the RIA. No significant cross-reactivities were observed indicating that chromatographic purification of PAF is not necessary prior to examination in the RIA. Lipid extraction, however, is still desirable since it eliminates effects due to the non-homogeneity of biological samples and PAF-binding proteins such as albumin that may be present. Extraction is also useful in order to concentrate the analyte in cases where normal PAF levels are too low to be measured.

To test the applicability of the RIA, PAF levels in a sample of normal human saliva were quantified. Saliva was chosen since it is reported that it contains PAF (Cox et al., 1981) and is readily obtainable. When quantified by platelet aggregation following HPLC purification, PAF levels in saliva were found to be very low (for example, < 2 pg/ml) (Wardlow, 1985). By RIA, we found the PAF content to be much higher (3.2 ng/ml). Studies are now being undertaken to determine salivary PAF levels in a larger population. There appears to be no interference when measuring saliva extracts by RIA since the expected values were obtained when the sample was diluted and when extra PAF was added.

The sensitivities of the RIA and the platelet aggregation assay were found to be similar and these assays are both about ten times as sensitive as platelet degranulation procedures. The RIA offers the advantages of being reproducible and simple to perform, so that numerous samples can be processed at any one time. The platelet-based assays require a lengthy preparation time, are difficult to standardise and generally only purified PAF should be used due to the possible presence in lipid extracts of potential agonists and/or inhibitors of platelet aggregation. Accurate quantitation of PAF by this method is difficult because of the variable nature of the aggregation response. A recent improvement to platelet-based procedures has been the use of ^{3}H-PAF in radioreceptor binding assays, utilizing either whole canine platelets (Janero et al., 1988) or rabbit platelet membranes (Paulson and Nicholson, 1988). These assays are based on the displacement of tracer PAF from the PAF-receptor complex by cold PAF and offer increased reproducibility over the older methods. They do, however, suffer from the disadvantage of high non-specific binding and have the potential for detecting substances other than PAF which bind to the receptor. The sensitivities of the receptor-based assays are similar to the RIA, ranging down to 10–20 pg.

In summary, the assay described here should be applicable to a wide variety of biological samples. Since extraction (usually by the Bligh-Dyer method) is generally likely to be the only preparation required, the procedure has a high capacity. Sensitivity is likely to be sufficient for most samples and can also be increased by extraction. Using this assay it should be possible to examine rapidly large numbers of clinical samples such as blood, urine, saliva, sputum and various lavage fluids. Hence, a clearer understanding of the role of PAF in health and disease should emerge in the near future.

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References

Barnes, P.J., Chung, K.F. and Page, C.P. (1988) Platelet activating factor as a mediator of allergic disease. J. Allergy Clin. Immunol. 81, 919.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. Canad. J. Biochem. Physiol. 37, 911.

Braquet, P., Mencia-Huerta, J.M., Chabier, P.E., Touqui, L. and Vargaftig, B.B. (1987) The promise of platelet activating factor. ISI Atlas Sci.: Pharmacol. 187.

Cox, C.P., Wardlow, M.L., Jorgensen, R. and Farr, R.S. (1981) The presence of platelet-activating factor (PAF) in normal human mixed saliva. J. Immunol. 127, 46.

Hanahan, D.J. (1986) Platelet activating factor: a biologically active phosphoglyceride. Ann. Rev. Biochem. 55, 483.

Hanahan, D.J. and Kumar, R. (1987) Platelet activating factor; chemical and biochemical characteristics. Prog. Lipid Res. 26, 1.

Hanahan, D.J. and Wientraub, S.T. (1985) Platelet activating factor; isolation, identification and assay. Methods Biochem Anal 31, 195.

Hanahan, D.J., Demopoulos, C.A., Liehr, J. and Pinckard, R.N. (1980) Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. J. Biol. Chem. 255, 5514.

Janero, D.R., Burghardt, B. and Burghardt, C. (1988) Radioligand competitive binding methodology for the evaluation of platelet-activating factor (PAF) and PAF-receptor antagonism using intact canine platelets. J. Pharmacol. Methods 20, 237.

Mallet, A.I. and Cunningham, F.M. (1985) Structural identification of platelet activating factor in psoriatic scale. Biochem. Biophys. Res. Commun. 126, 192.

Mueller, H.W., O'Flaherty, J.T. and Wykle, R.L. (1984) The molecular species distribution of platelet-activating factor synthesized by rabbit and human neutrophils. J. Biol. Chem. 259, 14554.

Paulson, S.K. and Nicholson, N.S. (1988) A radioreceptor binding assay for measurement of platelet-activating factor synthesis by human neutrophils. J. Immunol. Methods 110, 209.

Pettipher, E.R., Higgs, G.A. and Henderson, B. (1987) PAF-acether in chronic arthritis. Agents Actions 21, 98.

Prevost, M.-C., Cariven, C. and Chap, H. (1988) Possible origins of PAF-acether and lyso-PAF-acether in rat lung alveoli secondary to hypoxia. Biochim. Biophys. Acta 962, 354.

Ramesha, C.S. and Pickett, W.C. (1987) Species-specific variations in the molecular heterogeneity of the platelet activating factor. J. Immunol. 138, 1559.

Satouchi, K., Oda, M., Yasunaga, K. and Saito, K. (1985) Evidence for production of 1-acyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine concomitantly with platelet-activating factor. Biochem. Biophys. Res. Commun. 128, 1409.

Smal, M.A., Baldo, B.A. and Redmond, J.W. (1989) Production of antibodies to platelet activating factor. Mol. Immunol. 26, 711.

Snyder, F. (1985) Chemical and biochemical aspects of platelet activating factor: a novel class of acetylated ether-linked choline-phospholipids. Med. Res. Rev. 5, 107.

Tokumura, A., Kamiyasu, K., Takauchi, K. and Tsukatani, H. (1987) Evidence for existence of various homologues and analogues of platelet activating factor in a lipid extract of bovine brain. Biochem. Biophys. Res. Commun. 145, 415.

Vargaftig, B.B. and Braquet, P.G. (1987) PAF-acether today – relevance for acute experimental anaphylaxis. In: D.A. Willoughby (Ed), British Medical Bulletin. Vol. 43. Churchill Livingstone, London, p. 312.

Wardlow, M.L. (1985) Rapid isocratic procedure for the separation of platelet-activating factor from phospholipids in human saliva by high-performance liquid chromatography. J. Chromatogr. 342, 380.